Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editors: Vinood B. Patel
Victor R. Preedy

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Vinood B. Patel
Victor R. Preedy *Editors*

Biomarkers in Diabetes

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Biomarkers in Disease: Methods, Discoveries and Applications

Series Editors

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In the past decade there has been a major sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. New analytical methods that have been used to identify markers of disease and is suggested that there may be as many as 40 different platforms. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Diabetes

With 139 Figures and 69 Tables

Springer

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Preface

In recent years, there have been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies, as well as advances in chemistry and physics. This has led to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these progressions have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics, these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidence-based text that addresses these issues. Hence the book ***Biomarkers in Disease: Methods, Discoveries, and Applications: Biomarkers in Diabetes***. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and links with treatments. It links conventional approaches with new platforms.

In the present book, ***Biomarkers in Diabetes***, we have sections on:

1. ***Circulating and body fluid biomarkers***
2. ***Micronutrients and minerals***
3. ***Diets and macronutrients***
4. ***Genetic, molecular, and cellular variables***
5. ***Functional and physiological variables and platforms***
6. ***Biomarkers in specific conditions or scenarios***
7. ***Resources***

The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Editors recognize the difficulties in assigning chapters to parts of the book, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage and is well indexed.

The chapters are written by national and international experts. This book is designed for endocrinologists, clinical biochemists, health scientists specializing in diabetes, epidemiologists, researchers, doctors, and nurses, from students to practitioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and academic libraries as a reference guide.

London, UK
November 2022

Dr Vinood B. Patel
Professor Victor R. Preedy

Contents

Part I General Aspects 1

1 **Linking Variants of Hemoglobin A1C and Glycemic Status** 3
Jee-Young Moon and Qibin Qi

2 **Treatment Regimes in Diabetes and Their Impact on Biomarkers** 21
João P. Magalhães, Megan Hetherington-Rauth, and Luís Bettencourt Sardinha

Part II Circulating and Body Fluid Biomarkers: Impact of Diabetes on Pathology 65

3 **Resistin as a Biomarker and Applications to Prediabetes** 67
Seyfettin Üstünsoy

4 **Altered Metabolome of Amino Acids Species: A Source of Signature Early Biomarkers of T2DM** 83
Anna Czajkowska, Ahsan Hameed, Mauro Galli Muhammad Umair Ijaz, Adam Kretowski, and Michal Ciborowski

5 **Components of the Purine Metabolism Pathways as Biomarkers for the Early Diagnosis of Diabetes** 127
Ahsan Hameed, Mauro Galli, Anna Czajkowska, Adam Kretowski, and Michal Ciborowski

6 **Serpin A12 (Vaspin) as a Serine Protease Inhibitor** 153
Armin Salek Maghsoudi, Shokoufeh Hassani, Kayvan Mirnia, and Mohammad Abdollahi

7 **Advanced Glycation End Products in Diabetes** 171
Jesús A. Mosquera-Sulbarán and Juan Pablo Hernández-Fonseca

8 **Markers of Liver Function and Insulin Resistance** 195
Vicente Aleixandre Benites-Zapata, Sofía Lorena Bohórquez-Medina, and Andrea Lisbet Bohórquez-Medina

9 **Biological Markers of Insulin Sensitivity Links with Dietary Antioxidant** 217
Andrea Lisbet Bohórquez-Medina, Sofía Lorena Bohórquez-Medina, and Vicente Aleixandre Benites-Zapata

10 **Serum Uric Acid and Metabolic Markers in Diabetes** 239
Mohamed Rafiullah and Khalid Siddiqui

11 **Urinary Interleukins and Kidney Damage in Diabetes** 261
Rafael Noal Moresco, Guilherme Vargas Bochi, Manuela Borges Sangoi Cardoso, and José Antonio Mainardi de Carvalho

12 **Serum Homocysteine as a Biomarker in Diabetes** 279
Em Yunir and Yully Astika Nugrahayning Aziza

13 **3-Hydroxyisobutyrate (3-HIB): Features and Links as a Biological Marker in Diabetes** 299
Simon Nitter Dankel

14 **Proteomic Biomarkers: What They Are and How Type 2 Diabetes Mellitus Has Similarities with Other Diseases** 311
Karina Braga Gomes

15 **Salivary C-Reactive Protein as a Biomarker and Implications for Diabetes** 329
Vaithinathan Selvaraju, Jeganathan Ramesh Babu, and Thangiah Geetha

16 **Assessing Insulin Sensitivity in People with Type 1 Diabetes Without Euglycemic-Hyperinsulinemic Clamps** 349
Andrzej S. Januszewski and Alicia J. Jenkins

17 **Serum Betatrophin: What It Shows and How It Alters in Gestational Diabetes Mellitus** 375
Rauf Melekoglu and Ebru Celik

18 **Biomarkers in Disease: Diabetes Methods, Discoveries, and Applications** 395
Luiz Sérgio F. de Carvalho, Aline Ioshie Akamine Asari, Bruna Stefany Bento de Sousa Teles, Adriano de Britto Chaves Filho, and Marcos Yukio Yoshinaga

19 **Correlations Between Renal Biomarkers and the Treatment Outcomes in Diabetes: Ophthalmic Aspects** 411
Meng-Ju Tsai, Ivan Pochou Lai, Ming-Jui Lee, and Yi-Ting Hsieh

20 **Serum Paraoxonase 1 as a Biomarker: Features and Applications in Type 2 Diabetes Mellitus** 435
Berrin Öztaş, Fatma Ceyla Eraldemir, and Hale Maral Kır

21 The Ratio of Omega-6/Omega-3 Fatty Acid: Implications and Application as a Marker to Diabetes 449
Shilpa S. Shetty, N. Suchetha Kumari, and Remya Varadarajan

22 Engulfment and Cell Motility Protein (ELMO)-1 as a Biomarker in Type II Diabetes 469
Elfiani Elfiani, Anggelia Puspasari, and Zulkhair Ali

23 Brain-Derived Neurotrophic Factor and Vascular Endothelial Growth Factor A: Biomarkers Potential in Diabetes 485
Yulia Karakulova and Tamara Filimonova

24 The Role of Glycated Albumin as a Biomarker of Glycemic Control in Diabetes and Chronic Kidney Disease 513
Shanel Raghubeer, Don M. Matshazi, Dipuo D. Motshwari, Cecil J. Weale, Rajiv T. Erasmus, and Tandi E. Matsha

Part III Genetic, Molecular, and Cellular Variables **539**

25 Epigenetics and 5-Hydroxymethylcytosines as a Biomarker in Type 2 Diabetes 541
Chang Zeng and Wei Zhang

26 Adult Stem Cells as a Biomarker in Diabetes 559
Scott Cohen and Sabyasachi Sen

27 The Search for Immunological Biomarkers in Type 1 Diabetes Mellitus (T1DM) and Multiple Sclerosis (MS): Th40 Cells Provide a Common Autoimmune Link 575
David H. Wagner

28 Epigenetically Modified DNA Fragments 597
Sarah A. Tersey and Raghavendra G. Mirmira

29 Urinary Glucose Excretion as a Biomarker for Precision Medicine in Diabetes 615
Shinsuke Noso and Hiroshi Ikegami

30 Secreted Frizzled-Related Proteins 4 and 5: What They Are and Can They Be Used as a Biomarker in Gestational Diabetes Mellitus 629
Rik H. J. Beernink and Joost H. N. Schuitemaker

31 Insights into the World of MicroRNAs 649
Marta Greco, Eusebio Chiefari, Maria Mirabelli, Alessandro Salatino, Salvatore Andrea Pullano, Antonio Aversa, Daniela Patrizia Foti, and Antonio Brunetti

32 Genomic Ancestry as Biomarkers 669
Deborah Conte Santos

33 Hp1-1 as a Genetic Marker in Diabetes: Measures, Applications, and Correlations 681
Agnieszka Filipek

Part IV Functional Variables, Physiological Variables, and Platforms **703**

34 Day-by-Day Home Blood Pressure Monitoring as a Biomarker in Diabetes 705
Daisuke Suzuki, Satoshi Hoshide, and Kazuomi Kario

35 Methods for Measuring Blood Pressure and Applications to Diabetes 729
Andriani Vazeou and George S. Stergiou

36 Carotid Bodies: Use of Chemosensitivity as a Biomarker in Prediabetes 751
Silvia V. Conde, Marlene Lages, Maria P. Guarino, and Joana F. Sacramento

37 Optical Coherence Tomography Angiography for Biomarker Indices in Diabetes 777
Eun Young Choi and Min Kim

38 Urinary Profiling with Liquid Chromatography-Mass Spectrometry 791
Lay-Harn Gam

39 Physiologic Measures in Diabetes: QTc Prolongation 809
Lauren Ehrhardt-Humbert and Matthew J. Singleton

40 Biomarkers of Myocardial Fibrosis in Diabetes, Echocardiography, and Magnetic Resonance Imaging 821
Per Lav Madsen

41 Measures of Endothelial Function in Type 2 Diabetes: A Focus on Non-circulatory Methods of Measurement 849
Caroline Wei Shan Hoong

42 Measures of Endothelial Function in Type 2 Diabetes: A Focus on Circulatory Biomarkers 867
Caroline Wei Shan Hoong

Part V Biomarkers in Specific Conditions or Scenarios of Diabetes **889**

43 Biomarkers of Diabetes-Induced Nephropathy 891
Bamidele Stephen Ajilore and Bosede Olaitan Ajilore

44 Serum Vitamin D As a Biomarker in Diabetic: Applications and Associations with Retinopathy 909
Carolina Madeira and Manuel Falcão

45 Markers of Bacterial Translocation in Type 2 Diabetes Mellitus 923
Marwa Ahmed Meheissen

46 Serum Leptin as a Biomarker in Diabetes 947
Hind Shakir Ahmed

47 Vitreous Biomarkers: What They Are and How They May Be Used to Advance the Management of Diabetic Retinopathy 963
Ricardo Lamy and Jay M. Stewart

48 Protein Pyrrole Adducts in Diabetes Mellitus 991
Xiao Chen, Zhuyi Jiang, and Peter S. Spencer

49 Biomarkers in Gestational Diabetes 1021
Aykan Yucel and Betul Yakistiran

50 Metabolomic Biomarkers, Metabolite Patterns, and Gestational Diabetes Mellitus 1033
Ellen C. Francis and Wei Perng

51 Neuroinflammatory Biomarkers in Diabetic Encephalopathy: Linking Cholinergic and Cognitive Dysfunction 1053
Tosin A. Olasehinde, Sunday I. Oyeleye, Ademola O. Olaniran, and Md. Shahidul Islam

Part VI Diet and Diabetes **1073**

52 Selenium and Risk of Diabetes 1075
Shinje Moon and Chang-Myung Oh

Part VII Models and Modeling in Diabetes **1087**

53 Models of Diabetes in Rats: A Focus on Diabetic Neuropathy and Biomarkers 1089
Che Aishah Nazariah Ismail and Idris Long

54 Biomarkers in Experimental Diabetes: Studies with *Syzygium Cumini* (L.) and Links with the Sulfonylurea 1 Receptor 1111
Pratibha D. Nadig, Padmaja Shetty K, Anusha S. J, Meharban Asanaliyar, and Nagakumar Bharatam

Part VIII Resources **1139**

55 Recommended Resources for Biomarkers in Diabetes: Methods, Discoveries, and Applications 1141
Rajkumar Rajendram, Daniel Gyamfi, Vinood B. Patel, and Victor R. Preedy

Correction to: Assessing Insulin Sensitivity in People with Type 1 Diabetes Without Euglycemic-Hyperinsulinemic Clamps C1
Andrzej S. Januszewski and Alicia J. Jenkins

Index 1155

About the Editors

Vinood B. Patel, BSc, PhD, FRSC, is currently Reader in Clinical Biochemistry at the University of Westminster. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for the diagnosis and prognosis of liver disease and understanding mitochondrial oxidative stress in Alzheimer's disease and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his PhD in protein metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized researcher and has several edited biomedical books related to the use or investigation of active agents or components as well as biomarkers. These books include *The Handbook of Nutrition, Diet, and Epigenetics*; *Biomarkers in Cancer*; *Biomarkers in Cardiovascular Disease*; and *Biomarkers in Liver Disease*. In 2014, Dr. Patel was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy, BSc, PhD, DSc, FRSB, FRSPH, FRCPath, FRSC, is Professor of Clinical Biochemistry and Pathology at King's College Hospital, Emeritus Professor of Nutritional Biochemistry at King's College London, and Visiting Professor at the University of Hull. Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London PhD in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then, he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public

Health and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the Cardiothoracic Institute, National Heart Hospital (part of Imperial College London), The School of Pharmacy (now Part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long-standing interest in biomarkers, especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 750 articles, which includes peer-reviewed manuscripts based on original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

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Part I

General Aspects

Linking Variants of Hemoglobin A1C and Glycemic Status

1

Jee-Young Moon and Qibin Qi

Contents

Introduction 4
Overview of Genome-Wide Association Studies of HbA1c 5
Functional Characterization of HbA1c-Associated Genetic Variants 7
Glycemic-Related Genetic Variants 8
Erythrocyte-Related Genetic Variants 9
Unclassified Genetic Variants 11
Erythrocyte-Related HbA1c Variants and Glycemic Status Screening 12
G6PD: rs1050828 and rs76723693 12
HBB-rs334 13
G6PD-rs1050828 and HBB-rs334 14
Cumulative Effect of Erythrocyte-Related Genetic Variants 15
Conclusion 15
Applications to Other Diseases or Conditions 15
Mini-Dictionary of Terms 16
Key Facts of HbA1c 16
Summary Points 17
Reference 17

Abstract

Hemoglobin A1C (HbA1c) has been used as a convenient screening test for the diagnosis of diabetes and prediabetes, requiring no fasting, as well as a reliable measure for monitoring the glycemic control in people with diabetes. As HbA1c is the measure of the fraction of glycated hemoglobin out of total hemoglobin in red blood cells, both blood glucose levels and blood cell conditions affect levels of HbA1c. Consistently, genome-wide association studies on HbA1c have identified multiple genetic loci, largely grouped into two separate pathways – via the

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glycemic pathway and via the erythrocyte pathway. Two genetic variants, *G6PD*-rs1050828 and *HBB*-rs334, specifically found in individuals with African ancestry, were noted for their relatively large effects on HbA1c via the erythrocytic pathway, compared to other genetic variants. The glycemic status of individuals carrying the HbA1c-lowering alleles might be underestimated by HbA1c levels, as lower HbA1c level is observed compared to their actual glycemic status due to their genetic influences on blood cell biology. In addition, several erythrocyte-related genetic variants of small effects may also have a combined impact on HbA1c in this regard. Thus, the influences of these non-glycemic-related variants need to be considered when the HbA1c test is performed to measure glycemic status.

Keywords

HbA1c · Type 2 diabetes · Diabetes diagnosis · Genetic variant · SNP · GWAS · Erythrocyte · Blood traits · RBC · Glucose · G6PD deficiency · Hemoglobin · Sickle cell trait · HbA1c recalibration

Abbreviations

2 hr. OGTT	2-hour oral glucose tolerance test
ADA	American Diabetes Association
G6PD	Glucose-6-phosphate dehydrogenase
GRS	Genetic risk score
GWAS	Genome-wide association study
HbA1c	Hemoglobin A1C
MAF	Minor allele frequency
MAGIC	The Meta-Analysis of Glucose and Insulin-related Traits Consortium
MCV	Mean corpuscular volume
RBC	Red blood cell
SCT	Sickle cell trait
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
WGS	Whole genome sequencing

Introduction

Hemoglobin A1C or glycated hemoglobin (HbA1c) has been used for diabetes diagnosis as well as for monitoring glycemic control over the past 3 months for people with diabetes (American Diabetes Association 2010). This glycated hemoglobin is formed by blood glucose attached to the hemoglobin, specifically, N-terminal of beta-chains, in red blood cells (RBC, erythrocyte) by the non-enzymatic and irreversible reaction. Hence, the fraction of glycated hemoglobin out of total hemoglobin reflects the blood glucose levels over the lifespan of

erythrocytes, typically 120 days. While HbA1c indirectly measures blood glucose levels, HbA1c is a robust indicator of chronic hyperglycemia (high blood sugar) over the past 3–4 months, compared to a glucose-level measurement at one time point from a fasting blood glucose test or an oral glucose tolerance test, which can vary depending on the activity levels, stress, and hormone levels (Bonora and Tuomilehto 2011). Another strength of HbA1c test is that it can be performed any time without requiring 8 h of fasting or waiting for 2 h for the oral glucose test. Furthermore, HbA1c is a prognostic marker of diabetic complication risk such as diabetic retinopathy, nephropathy, and neuropathy (Skyler 1996). On the other hand, HbA1c levels can be influenced by hematologic conditions such as hemoglobinopathy, anemia, iron deficiency or overload, and recent blood loss or transfusion (Cohen et al. 2008; Sacks 2012; American Diabetes Association 2021).

Given this clinical importance of HbA1c, recent genome-wide association studies (GWASs) have been conducted to understand the genetics of HbA1c. These genetic studies suggest two major pathways on how genetic variants influence HbA1c levels. One is glycemic-related pathway and the other is erythrocytic-related pathway. We will look in detail on the identified genetic associations with HbA1c and describe the clinical implications of these findings.

Overview of Genome-Wide Association Studies of HbA1c

Since the first GWAS of HbA1c in 2007 (Meigs et al. 2007) until now in 2021, over 15 GWASs have been conducted on HbA1c by single ancestral group analysis in Europeans (Meigs et al. 2007; Pare et al. 2008; Franklin et al. 2010; Soranzo et al. 2010; An et al. 2014; Prins et al. 2017), Hispanics/Latinos (Moon et al. 2019; Wojcik et al. 2019), and East Asians or South Asians (Ryu and Lee 2012; Chen et al. 2013; Chen et al. 2014; Hachiya et al. 2017; Kanai et al. 2018; Spracklen et al. 2018; Chai et al. 2020) as well as by trans-ancestral analysis (Wheeler et al. 2017; Chen et al. 2021; Sarnowski et al. 2019) (Table 1). Very recently, the Meta-Analysis of Glucose and Insulin-related Traits Consortium (MAGIC) published the most diverse and 1 of the largest GWASs on HbA1c of 215,977 individuals free of diabetes from 76 cohorts (including majority of prior GWAS cohorts), comprised of 70% Europeans, 13% East Asians, 7% Hispanics/Latinos, 6% African Americans, 3% South Asians, and 2% sub-Saharan Africans (Chen et al. 2021). The study identified 127 HbA1c-associated loci (218 variants), explaining about 4.5–6% of HbA1c variation (Chen et al. 2021). In addition, a whole genome sequencing (WGS)-based association study identified several low-frequency or rare variants associated with HbA1c (i.e., rs1039215 in HBG2 and HBE1, rs76723693 in coding region of G6PD with p.Leu353Pro) in 10,338 individuals (6158 Europeans, 3123 Africa Americans, 650 Hispanics, and 407 East Asians) in the Trans-Omics for Precision Medicine (TOPMed) Program (Sarnowski et al. 2019). The analysis with WGS is expected to grow and provide enhanced understanding of genetics of HbA1c.

Table 1 Genome-wide association studies on HbA1c. Studies in bold face have functional characterization of identified genetic variants, which we refer often in this chapter. As GWAS studies are progressing to include more samples and diverse ancestry, large multi-cohort studies may include the study samples used in previously published paper. Most of summary are generated from GWAS Catalog, https://www.ebi.ac.uk/gwas/efotraits/EFO_0004541. Abbreviations: AA (African American), AF (African), EA (East Asian), EUR (European), HL (Hispanic/Latino), NR (Not reported), SA (South Asian), SEA (South East Asian)

First author (publication year)	Discovery population	Identified genetic loci
Meigs JB (2007) (Meigs et al. 2007)	982 EUR	4
Paré G (2008) (Pare et al. 2008)	14,618 EUR	4
Franklin CS (2010) (Franklin et al. 2010)	1782 EUR	1
Soranzo N (2010) (Soranzo et al. 2010)	46,368 EUR	11
Ryu J (2012) (Ryu and Lee 2012)	4275 EA	1
Chen P (2013) (Chen et al. 2013)	3427 EA, 1735 SEA, 1529 SA	3
An P (2013) (An et al. 2014)	4088 EUR	1
Chen P (2014) (Chen et al. 2014)	17,290 EA, 1727 SEA,	12
Prins BP (2017) (Prins et al. 2017)	9436 EUR	2
Wheeler E (2017) (Wheeler et al. 2017)	88,355 EUR, 7564 AA, 7572 SA, 18,472 EA	60
Hachiya T (2017) (Hachiya et al. 2017)	7704 EA	7
Kanai M (2018) (Kanai et al. 2018)	42,790 EA	28
Spracklen CN (2018) (Spracklen et al. 2018)	6943 EA	1
Moon JY (2019) (Moon et al. 2019)	9636 HL	10
Wojcik GL (2019) (Wojcik et al. 2019)	10,408 HL, 92 EA, 115 NR, 559 AA	20
Sarnowski C (2019)[a] (Sarnowski et al. 2019)	6158 EUR, 3123 AA, 650 HL, 407 EA	5
Chai JF (2020)[b] (Chai et al. 2020)	2704 SEA	4
Sinnott-Armstrong N (2021) (Sinnott-Armstrong et al. 2021)	327,177 EUR, 4847 AA, 6895 SA	470
Chen J (2021) (Chen et al. 2021)	215,974 subjects: AA (6%), EA (13%), EUR (70%), HL (7%), SA (3%), sub-Saharan African (2%)	127

[a]Whole genome sequencing-based GWAS
[b]GWAS array and whole exome sequencing-based GWAS

Functional Characterization of HbA1c-Associated Genetic Variants

As HbA1c level is influenced by both blood glucose level and hematologic conditions, GWASs of HbA1c also unveil the glycemic-related pathway and the erythrocyte-related pathway for these HbA1c loci (Fig. 1). The most recent work

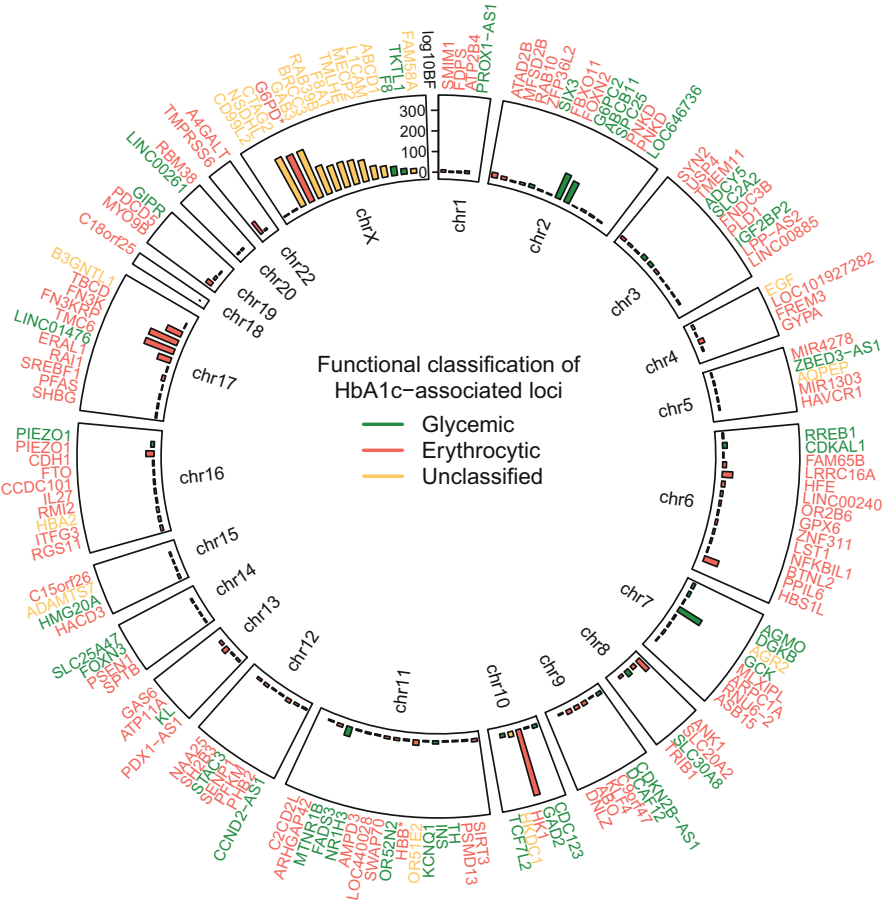

Fig. 1 HbA1c-associated genetic loci and functional classification. The loci and functional classification are from Supplemental Tables 2 and 20 in Chen et al. (2021). The erythrocyte-related classification includes the functional classification into mature red blood cell, reticulocyte, and iron-related classification. One genetic variant of the highest BF for each loci and classification was depicted in the figure. Log10BF is $\log_{10}$*Bayes Factor* of meta-analysis of single-ancestry GWAS using MANTRA, where log10BF > 6 considered to be genome-wide significant, approximately comparable to $P < 5 \times 10^{-8}$. *G6PD and HBB were not included for functional classification in Chen et al. (2021), but multiple studies indicate them to be associated with HbA1c via the erythrocyte pathway

lead by Chen et al. in MAGIC (Chen et al. 2021) classified 218 HbA1c-associated genetic variants into glycemic-related (n = 53); erythrocyte-related (n = 115) including mature red blood cell-related (n = 64), reticulocyte-related (n = 39), and iron-related (n = 12); and unclassified (n = 23), excluding 27 variants with insufficient information. This classification showed 82.1% consistency with a previous classification of 61 HbA1c-associated variants into 19 through the glycemic pathway, 22 through the erythrocytic pathway, and 19 unclassified, in the work by Wheeler et al. (2017).

The functional classification of the identified genetic variants have been performed by examining the summary association statistics of glycemic-related traits and erythrocyte-related traits (Wheeler et al. 2017; Moon et al. 2019; Soranzo et al. 2010; Sarnowski et al. 2019), by examining if the genetic association with HbA1c is attenuated after conditioning on glycemic- or erythrocyte-related traits (Wheeler et al. 2017; Soranzo et al. 2010), or by clustering the summary association signals of glycemic and erythrocyte traits using a non-negative matrix factorization (Chen et al. 2021). Glycemic traits include fasting glucose, 2-h glucose, fasting insulin, HOMA-B, or HOMA-IR (Wheeler et al. 2017; Moon et al. 2019; Chen et al. 2021). Erythrocyte-related traits include mature red blood cell traits, reticulocyte traits, and iron traits, which can affect the hemoglobin levels and the opportunity of glycation of hemoglobin (Wheeler et al. 2017; Moon et al. 2019; Chen et al. 2021).

Glycemic-Related Genetic Variants

These glycemic-related HbA1c variants are located in or near the genes which are known to be involved in glucose metabolism. For example, *GCK* encodes glucokinase, an enzyme sensing glucose, when blood glucose rises, the glucokinase helps to stimulate the insulin secretion from β-cells in pancreas (Matschinsky 1996). Multiple genetic variants (rs1799884 (in the promoter), rs2908286 (intron), rs2971670 (intron), rs3757840 (intron)) in the region of glucokinase gene (*GCK*) are associated with HbA1c and other glycemic traits (Wheeler et al. 2017; Chen et al. 2021; Soranzo et al. 2010). Given the important role of GCK in glucose metabolism, variants and mutations in *GCK* show a broad spectrum of glycemic disorders such as neonatal, childhood-onset, maturity-onset, and type 2 diabetes (Raimondo et al. 2014; Bonnefond et al. 2020; Vaxillaire et al. 2008; Fu et al. 2013). Yet, a clear mechanism remains unknown how the identified SNPs in the promoter or intron of *GCK* affect the functionality of GCK.

Another glycemic-related genetic variant is rs560887 (G-to-A) located in the intron of *G6PC2* gene, encoding glucose-6-phosphatase catalytic subunit-related protein (Soranzo et al. 2010; Wheeler et al. 2017; Chen et al. 2021). The gene is expressed specifically in pancreatic islets, and A allele of rs560887 is strongly associated with lower fasting glucose level, β-cell function (HOMA-B), and HbA1c (Bouatia-Naji et al. 2008). It has been suggested that G6PC2 plays a role in the glucose phosphorylation pathway, along with GCK and GCKR (glucokinase regulatory protein) (Bouatia-Naji et al. 2008). Other genetic loci affecting HbA1c

through the glycemic pathway include *CDKAL1, KCNQ1, INS, TCF7L2, MTNR1B, ABCB11,* and so on (Chen et al. 2021; Leong and Meigs 2015). A genetic risk score of 19 glycemic-related variants weighted by their effect sizes on HbA1c in Wheeler et al. was found to be associated with increased risk of incident diabetes during 10–15 years of follow-up (odds ratio per weighted allele 1.05, 95% CI 1.04–1.06) (Wheeler et al. 2017). In addition, another weighted genetic risk score of LD-pruned 53 variants in Chen et al. also showed the positive association with odds of T2D (Chen et al. 2021). These results support the validity of the classified glycemic-related genetic variants of HbA1c.

Erythrocyte-Related Genetic Variants

A well-established locus affecting HbA1c via erythrocyte-related pathway is *G6PD* (glucose-6-phosphate dehydrogenase) (Wheeler et al. 2017; Moon et al. 2019). G6PD is a key enzyme in producing NADPH (nicotinamide adenine dinucleotide phosphate), which prevents reactive oxygen species (ROS) to be built to toxic level within red blood cells. When an excess amount of ROS is triggered by certain drugs, infection, or fava bean intake to the people with G6PD deficiency, the damage and destruction of RBC is faster than its production, causing hemolytic anemia (MedlinePlus). This shortened lifespan of RBC, in other words, shorter period of time for hemoglobin to be glycated, leads to lower HbA1c level, which is observed for the people with *G6PD* deficiency. Consistently, the top signal rs1050828 (C-to-T for p.Val98Met) in *G6PD* was identified to be associated with HbA1c levels by several studies (Wheeler et al. 2017; Moon et al. 2019; Sarnowski et al. 2019), and individuals carrying minor allele (A allele) of rs1050828 showed lower HbA1c level, 0.81% units lower in hemizygous men (AA vs GG) and 0.68% units lower in homozygous women (AA vs GG) in African Americans (Wheeler et al. 2017), and 0.35% lower per A allele in US Hispanics/Latinos population (Moon et al. 2019), summarized in Table 2. In addition, this non-glycemic effect of rs1050828 on HbA1c is supported by the fructosamine measurement which reflects the serum protein glycation over 2–3 weeks, and the measured HbA1c is lower than fructosamine-predicted HbA1c for individuals carrying A allele (Wheeler et al. 2017). Further, a recent whole genome sequencing study identified several additional missense variants in *G6PD* such as rs76723693 (A-to-G for p.Leu353Pro, G allele frequency 0.5% in African ancestry, and 0.07% in Hispanics/Latinos) and rs5030872 (T-to-A for p.Asp211Val, MAF = 0.0002) (Sarnowski et al. 2019). Some of these variants in *G6PD* may have arisen from the positive natural selection against malaria infection as G6PD deficiency shows a relative protection against severe malaria (Guindo et al. 2007; Mason et al. 2007). This can be potentially explained by the rapid destruction of the infected red blood cells along with malaria parasite in people with G6PD deficiency, as the excessive ROS is produced in the red blood cell when hemoglobin is degraded by malaria (Mason et al. 2007; Cappadoro et al. 1998).

Another example is *HBB*-rs334 (A-to-T for p.Glu7Val), a causal mutation for sickle cell trait (SCT) and sickle cell disease (Lacy et al. 2017; Moon et al. 2019;

Table 2 Identified HbA1c-associated genetic variants in erythrocyte pathway with large effect sizes (~0.3% per allele). Abbreviations: AA (African American), HL (Hispanic/Latino)

SNP ID	Gene	Chr	Position (bp), GRCh38	Effect/ ref allele	Effect allele frequency[a]					First author (year)	Race	Effect size (SE) per one effect allele, %[b]
					EU	AA	HL	EA	SA			
rs1050828	G6PD	X	154,536,002	T/C	0.0002	0.116	0.012	0	0.0004	Wheeler et al. (2017)	AA	Men: −0.40 (0.08) Women: −0.34 (0.07)
										Sarnowski et al. (2019)	AA	Men: −0.44 (0.02) Women: −0.34 (0.23)
										Sarnowski et al. (2019)	HL	Men: −0.42 (0.10) Women: −0.25 (0.1)
										Moon et al. (2019)	HL	−0.35 (0.01)
										Chen et al. (2021)	AA	−0.31 (0.01)
										Chen et al. (2021)	HL	−0.28 (0.01)
										Chen et al. (2021)	African	−0.18 (0.01)
rs76723693	G6PD	X	154,533,025	G/A	0	0.005	0.002	0	0	Sarnowski et al. (2019)	AA	Men: −0.49 (0.12) Women: −0.46 (0.1)
rs334	HBB	11	5,227,002	A/T	0.0001	0.043	0.006	0	0.002	Moon et al. (2019)	HL	−0.31 (0.02)
										Lacy (2019)[c]	AA	−0.3 (0.05)

[a]From gnomAD v3.1.1

[b]Genotype in X chromosome is coded as 0 with a reference allele and 2 with an effect allele in men

[c]Non-GWAS study

Gordon et al. 2020; Chen et al. 2021). T allele is specifically found in African ancestry (4.3%) and Hispanics/Latinos (0.6%) (Table 2). The missense mutation of rs334 (p.Glu7Val) makes an abnormal type of hemoglobin, called hemoglobin S. Having two copies of hemoglobin S (TT genotype) makes rigid and sickle-shaped RBCs, called sickle cell disease, causing lifelong chronic hemolytic anemia. Individuals with heterozygous genotype (AT), classified to have SCT, don't show any symptoms like sickle cell disease, but can develop alike symptoms in rare cases under extreme circumferences like low oxygen level and increased pressure. It remains unclear why the people with SCT have lower HbA1c. A potential explanation is shorter lifespan of RBC with SCT, leading to less opportunity for glycation (Lacy et al. 2017), though there is limited and conflicting evidence (Gordon et al. 2020; Suarez et al. 1959; McCurdy 1969; Lacy et al. 2017). Another explanation is the smaller size of RBC (microcytes) with SCT, characterized by low mean corpuscular volume (MCV) (Moon et al. 2019). The small size puts RBCs more susceptible to ROS damage, leading to shorter lifespan (Vives Corrons et al. 1995). Lastly, a minor interference by hemoglobin S type in HbA1c measurement has been reported in certain HbA1c assays (Lacy et al. 2017; Roberts et al. 2005; Rohlfing et al. 2017), although the studies identifying the association between rs334 and HbA1c (Moon et al. 2019; Lacy et al. 2017) used HbA1c assays (Tosoh G7 and Tosoh 2.2) with no clinically significant interference (NGSP).

Other genetic loci influencing HbA1c through erythrocyte-related pathway include *FN3K, TMPRSS6, HK1, HFE*, and so on (Leong and Meigs 2015; Soranzo et al. 2010; Wheeler et al. 2017; Chen et al. 2021). In addition, Amerindian ancestry-specific variant, *HBM*-rs145546625, is suggested to affect HbA1c via RBC biology (Moon et al. 2019). Unlike the positive association between the glycemic-related GRS and incident diabetes, no association was observed between the erythrocyte-related GRS and incident diabetes (Wheeler et al. 2017). This corroborates two separate genetic pathways (i.e., glycemic and erythrocyte) influencing HbA1c levels. Furthermore, it suggests to consider erythrocyte-related genetic factors and/or non-glycemic health conditions which can influence HbA1c levels in a broad sense, in using HbA1c as a diagnostic marker for diabetes, as HbA1c levels can be increased or decreased depending on their blood-related genetic factors, unrelated with glycemic status.

Unclassified Genetic Variants

Although Chen and colleagues (2021) were able to classify 16 out of 19 unclassified genetic variants in Wheeler et al. (2017) mostly into erythrocyte related variants, aided by expanded GWAS on glycemic, RBC, and iron traits as well as the application of statistical methods for classification (e.g., a fuzzy clustering), 11% of HbA1c-associated variants are still unclassified such as *VPS13A*-rs7847351 and rs12351997, *SPTA1*-rs8577725 and rs857676, *FADS2*-rs174584, etc. (Chen et al. 2021; Wheeler et al. 2017). Further studies enhanced with large-scale, diverse population, WGS data, gene expression, and epigenome will help better understand mechanisms underlying these associations.

Erythrocyte-Related HbA1c Variants and Glycemic Status Screening

As erythrocyte-related genetic variants affect HbA1c level by hematologic pathway not via glucose metabolism pathway, the information on genetic variants can augment the HbA1c test for better diagnosis of diabetes and monitoring of glycemic control. Multiple research advocate this by examining the potential consequence after taking into account a few erythrocyte-related genetic variants with large effects (*G6PD*-rs1050828 and rs76723693 and/or *HBB*-rs334) (Wheeler et al. 2017; Lacy et al. 2017; Moon et al. 2019) or a cumulative effect of overall erythrocyte-related genetic variants (Soranzo et al. 2010; Moon et al. 2019). Table 2 summarizes the list of genetic variants estimated to have a large genetic effect of ~0.3% per allele on HbA1c to markedly interfere the clinical interpretation of HbA1c test, while other variants were estimated to have small effects on HbA1c around 0.03% per allele. For reference, the HbA1c cut-off suggested by the American Diabetes Association (ADA) is $\geq$6.5% (48 mmol/mol) for diabetes and 5.7–6.4% (39–47 mmol/mol) for impaired glucose tolerant/prediabetes (American Diabetes 2018). The clinical importance of HbA1c test for diabetes screening is emphasized by the convenience of test without fasting or waiting, despite the slightly lower sensitivity compared to fasting glucose or 2-h glucose in OGTT (Cowie et al. 2010; Selvin et al. 2011). The following examples illustrate the avenue for HbA1c test in consideration of genetic factors for better screening of diabetes, summarized in Table 3.

G6PD: rs1050828 and rs76723693

Multiple studies consistently showed a large effect of rs1050828 in *G6PD* on HbA1c levels (Table 2). Further, a WGS-based study identified an independent, large-effect rare variant rs76723693. Both variants were observed specifically in African ancestral populations such as 12% in African American and 2% in Hispanics for A allele of rs1050828 and 0.5% in African American for G allele of rs76723693 (Sarnowski et al. 2019). Sarnowski et al. (2019) estimated that if a single measurement of HbA1c was solely used for diabetes diagnosis without consideration of these two *G6PD* variants, 2.32% of people with HbA1c $<$ 6.5% would have missed to have diabetes in African Americans and 0.26% in Hispanics/Latinos due to *G6PD*-rs1050828 and additional 0.13% in African Americans due to *G6PD*-rs76723693, estimated from the National Health and Nutrition Examination Survey (NHANES) in 2015–2016. Thus, HbA1c tests with consideration of G6PD variants would diagnose additional 740,000 African American adults with 40,000 from the rare variant rs767723693 and 100,000 Hispanic adults to have diabetes, given 30.14 million African American adults and 39.33 million Hispanics adults from 2016 US Census Bureau (Sarnowski et al. 2019).

Table 3 Potential clinical implication of erythrocyte-related genetic variants on HbA1c test for diabetes screening, among the people without any prior physician diagnosis or anti-diabetic medication. The percentage of reclassification is shown comparing the diabetes diagnosis by HbA1c (≥6.5%) and by erythrocyte-related variants adjusted HbA1c (≥6.5%), unless otherwise noted. Abbreviations: AA (African American), EA (East Asian), EUR (European), HL (Hispanic/Latino)

Single or two variant adjustment	First author (Year)	% of people with reclassification from undiagnosed to diagnosed	
G6PD-rs1050828	Wheeler E (2017)	2% in AA	
G6PD-rs1050828 and rs76723693	Sarnowski C (2019)	2.32% adjusting for rs1050828 in AA + 0.13% with additional adjustment of rs76723693 in AA 0.26% adjusting for rs1050828 in HL	
G6PD-rs1050828 + HBB-rs334	Moon JY (2019)	1.3% for prediabetes or diabetes (HbA1c > 5.7%) in HL	
Multiple variant adjustment	**First author (year)**	**% of people with reclassification from undiagnosed to diagnosed[a]**	**% of people with reclassification from diagnosed to undiagnosed[a]**
7 SNPs (FN3K, HFE, TMPRSS6, ANK1, SPTA1, ATP11A, and HK1)	Soranzo N (2010)	0.02% in EUR	8.7% in EUR
6 SNPs (TMEN79, HB21L/MYB, MYO9B, CYBA, ANK1, and FN3K)	Chen P (2014)	0.29% in EA	29.2% in EA
22 SNPs (TMEM79, SPTA1, SYN2, HFE-rs1800562, HFE-rs198846, C6orf183, MYB, CITED2, ANK1, SLC20A2, C9orf47, HK1, CNTN5, SENP1, ATXN2, ITFG3, CDH3, CDT1, ERAL1, MYO9B, TMPRSS6, G6PD)	Wheeler E (2017)	0.04% in EUR 0.68% in AA 0.14% in EA	10.5% in EUR 18.1% in AA 8.5% in EA

[a]Reinterpreted the result from each paper

HBB-rs334

About 1 in 13 African Americans has SCT (Centers for Disease Control and Prevention), and individuals with AT genotype of *HBB*-rs334 (the most common mutation for SCT) have 0.29% lower HbA1c value (95% CI 0.23–0.35%) than those without SCT

(rs334 AA genotype), for the same fasting glucose or 2-h glucose levels (Lacy et al. 2017). Likewise, among individuals without self-reported diabetes or antidiabetic medication use, the prevalence of prediabetes and diabetes were comparable between *HBB*-rs334 genotype groups when prediabetes and diabetes were defined by fasting glucose or 2-h glucose. When HbA1c was used to define the hyperglycemia, individuals with AT genotype of *HBB*-rs334 (SCT) had lower prevalence in prediabetes (29.2% vs 48.6%) and diabetes (3.8% vs 7.3%) compared to those with AA genotype (no SCT).

G6PD-rs1050828 and HBB-rs334

In US Hispanics/Latinos excluding those with diagnosed diabetes or sickle cell disease (TT genotype of HBB-rs334), rs1050828 in *G6PD* and rs334 in *HBB* were both identified to have large effects on HbA1c: −0.35% per A allele of *G6PD*-rs1050828 and − 0.33% per T allele of HBB-rs334 (Moon et al. 2019). The prevalence of hyperglycemia (prediabetes and diabetes) defined using fasting glucose (≥ 100 mg/dL) or OGTT 2-h glucose (≥ 140 mg/dL) was similar between carriers of *HBB*-rs334 or *G6PD*-rs1050828 HbA1c-lowering alleles and non-carriers (21.2% vs 25.4% and 18.6% vs 21.8%, respectively), while the prevalence of hyperglycemia defined using HbA1c (≥ 5.7%) was significantly lower in carriers than non-carriers (12.2% vs 28.4%). Similarly, carriers tended to have lower HbA1c than non-carriers at the same fasting glucose levels (Fig. 2 left panel). After the recalibration of HbA1c taking into account both variants, $HbA1c_{recalibrated}$

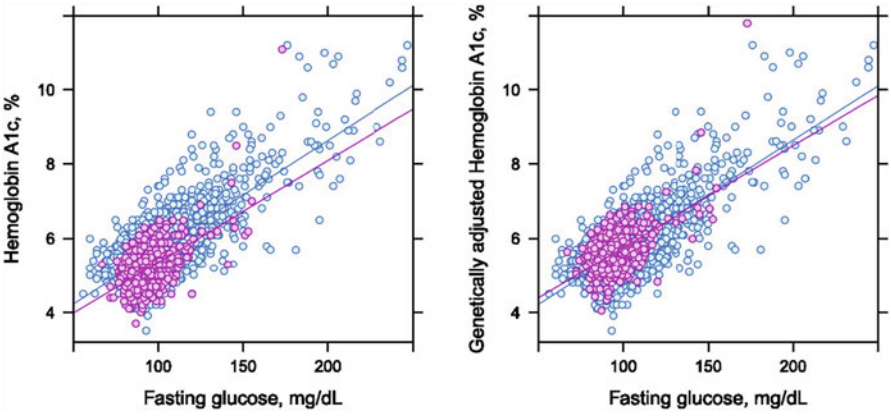

Fig. 2 Scatterplots of measured HbA1c and genetically adjusted HbA1c against fasting glucose in carriers and non-carriers of HBB-rs334 or G6PD-rs1050828 minor alleles. Zoomed-in plots among the individuals without diagnosed diabetes. Red points indicate carriers, and blue points indicate non-carriers of HBB-rs334 or G6PD-rs1050828 minor alleles. Published previously in Moon et al. A Genome-Wide Association Study Identifies Blood Disorder-Related Variants Influencing Hemoglobin A1c With Implications for Glycemic Status in U.S. Hispanics/Latinos. Diabetes Care 2019;42(9):1784–1791. Copyright 2019 by the American Diabetes Association

(%) = $HbA1c_{measured}$ (%) + 0.33 × number of T alleles in HBB-rs334 + 0.35 × number of A alleles in G6PD-rs1050828, the hyperglycemia prevalence defined by HbA1c was similar between carriers and non-carriers (31.3% vs 28.4%). Also, carriers and non-carriers showed comparable recalibrated HbA1c levels against the same fasting glucose levels (Fig. 2 right panel).

Cumulative Effect of Erythrocyte-Related Genetic Variants

Although the effect sizes of majority of erythrocyte-related genetic variants are small to make a clinical difference on its own, a cumulative effect of multiple erythrocyte-related genetic variants may also have a clinical influence on HbA1c levels. For example, between top 5% and bottom 5% of a weighted GRS of 22 erythrocyte-related genetic variants, the mean difference in HbA1c was 0.26% (95% CI 0.22–0.30%) in a European ancestry population (Wheeler et al. 2017). Thus, the cumulative effect of erythrocyte-related genetic variants could have a critical impact in diabetes screening using HbA1c. Adjusting for the effect of erythrocytic variants on HbA1c levels has been shown to improve reclassification for individuals with discordant diabetes status between HbA1c ($\geq$ 6.5% for diabetes) and fasting glucose ($\geq$ 7 mmol/L for diabetes) (Wheeler et al. 2017). Out of 18,613 individuals without diabetes by fasting glucose (<7 mmol/L), 266 individuals were classified to have diabetes by unadjusted (HbA1c ($\geq$ 6.5%) and 50 (18.8% reclassification) individuals were reclassified to have no diabetes using the adjusted HbA1c level with cumulative erythrocytic variants' effects. Among 390 individuals classified to have diabetes by fasting glucose but not by HbA1c, only 5 (1.3%) individuals were reclassified to have diabetes using adjusted HbA1c.

Conclusion

GWAS on HbA1c, accompanied by GWAS on glycemic traits and blood traits, illustrates two separate pathways for HbA1c – glycemic pathway and erythrocytic pathway. The genetic variants in the glycemic pathway along with GWAS on other glycemic traits will provide the comprehensive understanding of the development of diabetes. The erythrocyte-related genetic variants associated with HbA1c suggest the clinical use of recalibrated HbA1c by genetic variants for hyperglycemia diagnosis, especially, *G6PD*-rs1050828 and *HBB*-rs334. These studies are expected to merit the personalized strategy for the prevention, diagnosis, and treatment of diabetes.

Applications to Other Diseases or Conditions

In this chapter, we reviewed genetic variants associated with HbA1c levels identified by recent GWAS and their implications in hyperglycemia and diabetes diagnosis. In particular, several studies have suggested a potential clinical use of recalibrated HbA1c by erythrocyte-related genetic variants for hyperglycemia and diabetes diagnosis. It is

possible that these erythrocyte-related genetic variants may also have implications in other diseases or conditions, especially diabetes complications, such as cardiovascular disease, chronic kidney disease, neuropathy, etc. For example, Yeung et al. (2018) (Au Yeung et al. 2018) found that the glycemic-related GRS, but not the erythrocyte-related GRS, was associated with risk of CAD using the Mendelian randomization.

Mini-Dictionary of Terms

- ***G6PD deficiency.*** *An inherited condition through G6PD gene on the X chromosome, hence affecting more males than females. The people with G6PD deficiency do not have enough enzyme called G6PD (glucose-6-phosphate dehydrogenase), which protects red blood cells against reactive oxygen species. Hemolytic anemia (premature breakdown of red blood cells) can be triggered on the people with G6PD deficiency under certain conditions such as bacterial or viral infections, antimalarial medication, aspirin, nonsteroidal anti-inflammatory medication, and fava bean intake.*
- ***Genetic risk score (GRS).*** *A score for the susceptibility to a disease (trait), calculated based on the genetic variants and their effects associated with the disease (trait).*
- ***Genetic variant.*** *An alternative nucleotide sequence to the most commonly observed DNA nucleotide sequence. The impact of the variant varies – benign, uncertain significance, and pathogenic.*
- ***Genome-wide association study (GWAS).*** *A study to identify genetic variants associated with a particular trait across the genome.*
- ***Hemoglobin.*** *A protein molecule in red blood cells to transport oxygen from the lungs to the tissues in the body. The normal hemoglobin consists of two alpha-globins and two beta-globins. Each of the globins contains an iron-containing molecule, called heme, which binds to oxygen.*
- ***HbA1c.*** *The percentage of glycated hemoglobin. Glycated hemoglobin is formed by the hemologin by the spontaneous chemical binding reaction of a sugar such as glucose, galactose, and fructose.*
- ***Sickle cell trait.*** *The people with sickle cell trait carry one copy of a defective beta-globin gene, making an abnormal hemoglobin (HbS). The people with sickle cell trait do not show symptoms like the people with sickle cell disease with two copies of defective beta-globin gene, presenting sickle-shaped red blood cells.*
- ***Whole genome sequencing (WGS).*** *A next-generation sequencing technique to sequence the DNA (genome) base by base. In contrary, assay-based genotyping provides the genetic variation information for pre-determined set of genetic variants.*

Key Facts of HbA1c

HbA1c is the percentage of glycated hemoglobin out of total hemoglobin.
HbA1c is the indirect measure of blood glucose level over the lifespan of red blood cells, typically 120 days.

Independent of glucose level, HbA1c can be affected by hematological conditions such as lifespan of erythrocytes, iron deficiency, and blood transfusion.
An HbA1c test is widely used for diabetes screening test, requiring no fasting.
HbA1c level is used to monitor the glycemic control for people with diabetes.

Summary Points

- *HbA1c level is affected by blood glucose level and hematological conditions.*
- *GWAS identified multiple genetic loci associated with HbA1c,* via *glucose metabolism pathway and* via *erythrocytic pathway.*
- *Two African ancestry-specific variants, G6PD-rs1050828 and HBB-rs334, showed around 0.3% lower HbA1c per one minor allele, which poses clinical consideration of the two variants for HbA1c-based diabetes screening test.*
- *Multiple erythrocyte-related genetic variants of small size can also be considered to recalibrate HbA1c for diabetes screening test.*
- *Glycemic-related genetic variants infer the genetic susceptibility to diabetes.*

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Treatment Regimes in Diabetes and Their Impact on Biomarkers

2

Exercise, Inflammation, and Lipids

João P. Magalhães, Megan Hetherington-Rauth, and Luís Bettencourt Sardinha

Contents

Lipid Profile and T2DM 23
Introduction to Lipids and T2DM 23
The Relationship Between Obesity and Lipids 23
The Relationship Between Lipids and T2DM 25
Lipid Intermediates and Insulin Resistance 26
Role of Exercise on Lipid Metabolism in T2DM 28
Inflammatory Profile and T2DM 40
Introduction to Inflammation and T2DM 40
The Relationship Between Obesity and Inflammation 40
The Relationship Between Inflammation and T2DM 42
Anti-inflammatory Effects of Exercise 45
Applications to Other Diseases or Conditions 54
Conclusion 55
Mini-Dictionary of Terms 56
Key Facts on Exercise and the Lipid Profile 57
Key Facts on Exercise and Inflammation 57
Summary Points 57
References 58

Abstract

In our modern society, we are faced with a mismatch between our evolutionary past as a human species and the current challenges arising from a modern society, where physical inactivity and the consumption of energy-dense foods are indulged and ubiquitous. It is with no surprise that over the past century, this paradigm has led to an increase in the incidence and prevalence of several

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noncommunicable chronic diseases, including obesity, cardiovascular diseases, neurodegenerative diseases, cancer, and the special focus of this chapter, type 2 diabetes mellitus (T2DM), in which the lipid and inflammatory profiles play a major role in the pathophysiology.

In the following sections, we will provide an insight on how the lipid and inflammatory profiles are related to the pathophysiology of T2DM and shed some light on how acute and chronic exercise can impact and ameliorate the metabolic dysfunction in *T2DM individuals*. In the first section, we will address the relationship of the lipid biomarkers with both obesity and T2DM by examining the classic pathophysiology of dyslipidemia, while going into further detail on the organ-crosstalk among the adipose tissue, muscle, and liver. At the muscle level, we will highlight the interrelationships between intramyocellular lipids and lipid intermediates and the development of muscle insulin resistance, whereas, at the liver, we will discuss how intrahepatic lipids are a strong lipid biomarker capable of predicting hepatic insulin resistance and other obesity-related complications. We will then connect how acute and chronic exercise can impact these aforementioned lipid biomarkers through a mechanistic approach, as well as provide a detailed review of the most recent results from randomized controlled trials (RCT) on the classic, clinically used lipid biomarkers (e.g., low- and high-density lipoprotein cholesterol and triglycerides). In the second section, we will review the link between adipose tissue dysfunction and chronic low-grade inflammation in both obesity and T2DM, while taking a closer look at the inflammatory biomarkers that have been mostly studied in the exercise physiology field and that have been implicated in the pathophysiology of T2DM. Finally, and following the same trend as the first section on lipid biomarkers, we will discuss the impact of acute and chronic exercise on the inflammatory milieu and review the most recent RCT investigations, while focusing on the impact of certain exercise characteristics, such as the duration, intensity, and type of intervention.

Keywords

Dyslipidemia · Obesity · Insulin resistance · Aerobic · Resistance · Cytokines · Adipokines · Myokines · Lipid intermediates · Randomized controlled trials

Abbreviations

CRP	C-reactive protein
DAG	Diacylglycerols
DNL	De novo lipogenesis
FFA	Free fatty acid
HDL-C	High-density lipoprotein cholesterol
HIIT	High-intensity interval training
IHL	Intrahepatic lipids
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IMCL	Intramyocellular lipids

LCA-CoA	Long-chain acyl-CoAs
LDL-C	Low-density lipoprotein cholesterol
LPL	Lipoprotein lipase
MCT	Moderate continuous training
NAFLD	Nonalcoholic fatty liver disease
RCT	Randomized controlled trial
RT	Resistance training
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
VLDL	Very-low-density lipoprotein

Lipid Profile and T2DM

Introduction to Lipids and T2DM

Fat and lipids are essential components of all cells of the human body, where they play an important role in energy production, cell membrane structure, as well as many signaling processes. However, under circumstances of sustained positive energy balance, excessive circulating lipids and fat storage can lead to the increased risk of several chronic diseases, including T2DM. The energy balance mismatch is a common occurrence in our modern society and is considered the main culprit behind the overload of the adipose tissue depots that results in the overspill of lipids into other organs (i.e., ectopic fat), especially that of the muscle and liver, which contributes to insulin resistance in these tissues and the development of T2DM. In the following sections ("The Relationship Between Obesity and Lipids," "The Relationship Between Lipids and T2DM," and "Lipid Intermediates and Insulin Resistance"), we intend to provide an overview of the role of lipids in obesity and T2DM, while further exploring the relationship between intramyocellular lipids, lipid intermediates, and intrahepatic lipids with the development of insulin resistance and other obesity-related complications. We will then go from mechanisms to orientation on how acute and chronic exercise can be used as a viable strategy to improve these lipid-related biomarkers and contribute to an improved treatment and control of T2DM in section "Role of Exercise on Lipid Metabolism in T2DM."

The Relationship Between Obesity and Lipids

Obesity is at the heart of several cardiometabolic dysfunctions, including but not limited to, impaired glucose tolerance, hypertension, and dyslipidemia (Tchernof and Despres 2013; Heymsfield and Wadden 2017). The first evidence that individuals presenting a visceral adipose tissue phenotype had an impaired metabolic profile came in the late 1980s, where higher triglyceride and plasma glucose levels were observed following an

oral glucose test when compared with matched body mass index (BMI) peers with an abdominal subcutaneous adipose tissue phenotype (Fujioka et al. 1987). Following this same line of research, several investigations laid down additional support that individuals with obesity and a visceral adipose tissue phenotype were considered at risk for several cardiometabolic risk factors, including dyslipidemia and impaired glycemic control, and hence at risk for developing T2DM (Despres et al. 1990; Ross et al. 2020). Moreover, these previous investigations also addressed the importance of looking beyond the quantity of body fat mass, and instead focusing on the quality of the adipose tissue, represented by the extensibility capacities of the abdominal subcutaneous and visceral adipose tissue, and their relationship with cardiometabolic risk factors (Tchernof and Despres 2013; Heymsfield and Wadden 2017).

When faced with a continuous energy surplus due to a positive energy balance, the subcutaneous adipose tissue will be confronted with circumstances where it may not be able to cope and expand. This, in turn, may lead to a lipodystrophic state and the accumulation of lipids in unsought sites such as the visceral cavity (Tchernof and Despres 2013, Heymsfield and Wadden 2017). In such a scenario, it is important to understand that visceral adipose tissue has different lipolytic responsiveness to insulin stimulation when compared with peripheral subcutaneous adipose tissue (Ross et al. 2020). In fact, when exposed to a high dose of insulin, subcutaneous adipose tissue in the lower body can suppress its lipolytic activity, whereas visceral adipose tissue can only suppress lipolysis by half under the same conditions (Nielsen et al. 2004). One possible explanation for the differing responsiveness to insulin may be related to adipocyte size, as insulin sensitivity is inversely related to fat cell size and the level of hypertrophy of the adipocyte (Bays 2011). The higher basal and stimulated rates of lipolysis found in the larger adipocytes characteristic of visceral fat can greatly impact the lipid profile, given that approximately 50% of the portal vein flux of free fatty acids (FFA) in individuals with obesity originates from visceral fat, whereas in lean individuals, this value is only around 5–10% (Nielsen et al. 2004).

Overall, the impaired capacity of properly storing lipids following a meal in addition to the increase in circulating FFA are responsible for the subsequent increase of fat deposited in the visceral tissues. Given that the liver plays a major role in regulating lipid transport, the accumulation of fat within this visceral tissue is a cornerstone of the dyslipidemia process (Petersen and Shulman 2018). Individuals with obesity and a fatty liver have an overproduction of apolipoprotein B (apoB) to accommodate the increased amount of triglycerides (TG) in the liver (derived from the increased flux of FFA), which are later incorporated into large very-low-density lipoproteins (VLDL) (Petersen and Shulman 2018). The combination of large VLDL particles and the inability of the insulin-resistant liver to suppress VLDL secretion postprandially are the culprits behind the hypertriglyceridemia state, which will be further detailed in the following section regarding lipids and T2DM. Other characteristics of dyslipidemia observed in the visceral adipose tissue phenotype include low values of high-density lipoprotein cholesterol (HDL-C), with a decrease in the number and size of these lipoproteins, and an increase in low-density lipoprotein cholesterol (LDL-C) that tends to be smaller and denser than those found in a healthier lipid profile (Tchernof and Despres 2013). These changes in LDL-C,

which are a consequence of the increased values of apoB and the changes observed in the VLDL particles, alongside the remaining dysfunctional lipid profile found in individuals with obesity, are responsible for the increased cardiometabolic risk with obesity (Tchernof and Despres 2013; Seidman et al. 2014).

The Relationship Between Lipids and T2DM

The mechanism of lipid-induced insulin resistance remains not fully elucidated. As previously explained, when the buffering capacity of adipose tissue to store fatty acids is exceeded, the overspill of lipids into other organs is expected, leading to the accumulation of ectopic fat. In particular, the storage and accumulation of lipids in the liver and muscle is a hallmark of T2DM (Petersen and Shulman 2018). It is when looking at this triangle between the adipose tissue, muscle, and liver that we are able to understand both tissue-autonomous and crosstalk-dependent mechanisms through which lipids can lead to an insulin-resistant state. For instance, in the liver, the increased circulating levels of FFA and glycerol derived from the insulin-resistant lipolytic adipose tissue are responsible for the impaired processes of glycogen synthesis and gluconeogenesis (Petersen and Shulman 2018). Both glycerol and pyruvate carboxylase (which is increased from FFA beta-oxidation) are responsible for increasing gluconeogenesis and hepatic glucose production (Despres et al. 1990).

Beyond the impact of lipids on insulin resistance, lipid accumulation in the liver can also lead to nonalcoholic fatty liver disease (NAFLD), a common comorbidity coexisting in the majority of individuals with T2DM (Brouwers et al. 2016). Intrahepatic lipid (IHL) accumulation can occur as a result of: (1) saturated VLDL-TG export, (2) increased uptake of FFA due to higher circulating concentrations resulting from elevated lipolysis from insulin-resistant adipose tissue, (3) a reduction in FFA oxidation, and (4) increased liver FFA synthesis, otherwise known as de novo lipogenesis (DNL), which is stimulated by hyperinsulinemia and hepatic glucose uptake (Brouwers et al. 2016). The selective insulin resistance observed in T2DM is a major contributor facilitating the accumulation of IHL and the development of NAFLD in individuals with T2DM. Although the actions of insulin to suppress lipolysis in adipose tissue and VLDL production by the liver through Fox01 gene are impaired in T2DM, insulin is still able to stimulate the mechanistic target of rapamycin complex 1 (mTORC1), which promotes DNL and apoB secretion (Taskinen and Boren 2015). As a result, there is an increased flux of FFA to the liver from adipose tissue lipolysis and DNL, which together expand the pool of IHL, promoting the increased secretion of apoB-VLDLs as well as FFA oxidation (Adiels et al. 2008; Taskinen and Boren 2015). Eventually, however, the increased secretion of VLDLs and rate of FFA oxidation are unable to keep up with the continuing increased flux of FFA to the liver, which results in further IHL accumulation leading to hepatic steatosis (Taskinen and Boren 2015). In fact, IHL is a strong lipid biomarker with a superior ability to predict hepatic insulin resistance and other obesity-related complications when compared with the overall amount of visceral adipose tissue.

At the muscle level, a similar mechanism can be observed on the accumulation of lipid content, with the adipose tissue lipolysis being responsible for the accumulation of intramyocellular lipids (IMCL) and the consequent lipid-induced muscle insulin resistance (Despres et al. 1990). However, it is not the total amount of IMCL that directly causes insulin resistance (Sokolowska and Blachnio-Zabielska 2019), but instead, it is the dynamics and characteristics of the lipid droplets where the IMCL TG content is stored, as well as the production and accumulation of lipid intermediaries derived from impaired lipid oxidation and lipolysis, that seem to be responsible for the lipid-induced insulin resistance (Petersen and Shulman 2018; Sokolowska and Blachnio-Zabielska 2019) (Fig. 1). These deleterious lipid intermediates include diacylglycerols (DAG), sphingolipids (e.g., ceramides), and polar lipids (e.g., long-chain acyl-CoAs (LCA-CoA) and acylcarnitine), which will be further explored in the following sections.

Lipid Intermediates and Insulin Resistance

In the attempt to identify which lipid biomarkers were related to insulin resistance, researchers have long focused on three major classes of lipid intermediates: polar lipids, DAG, and sphingolipids.

As part of the polar lipids, LCA-CoA is the first active lipid intermediate formed during FFA metabolism, with possible implications on the synthesis of other deleterious lipid intermediates (Ussher 2014). Within this same family, acylcarnitine is an intermediate generated during mitochondrial FFA oxidation in order to facilitate the transfer of LCA-CoAs across the mitochondrial membrane, which is impermeable to CoA esters. It is formed by the conversion of LCA-CoA and carnitine via carnitine palmitoyltransferase 1 (CPT-1) located on the outer mitochondrial membrane (Metcalfe et al. 2018). Once inside the mitochondrial matrix, acylcarnitine is converted back to its corresponding LCA-CoA and carnitine by carnitine palmitoyltransferase 2 (CPT-2) located on the inner mitochondrial membrane (Metcalfe et al. 2018). From there, the LCA-CoA undergoes β-oxidation, which breaks down the fatty acyl chain to acetyl-CoA. Fatty oxidation is completed with the entry of acetyl-CoA into the tricarboxylic acid (TCA) cycle to ultimately generate ATP. However, when the rate of β-oxidation exceeds that of the TCA cycle, which occurs with a sustained mitochondrial oversupply of lipids, there is incomplete oxidation of LCA-CoAs (Ussher 2014). These incompletely oxidized LCA-CoAs accumulate and are reconverted back to acylcarnitine by CPT-2 (Ussher 2014). It has been proposed that these acylcarnitines directly impair insulin signaling by interfering with protein kinase B (AKT) phosphorylation (Bosma et al. 2012). On the other hand, the reactive oxygen species (ROS)/acylcarnitine hypothesis may also explain how these biomarkers contribute to the development of insulin resistance. Incomplete FFA oxidation leads to ROS production and thus, acylcarnitine may serve more as a marker of incomplete FFA oxidation and, thus, a reflection of ROS levels than being directly related with insulin resistance (Bosma et al. 2012; Metcalfe et al. 2018; Petersen and Shulman 2018).

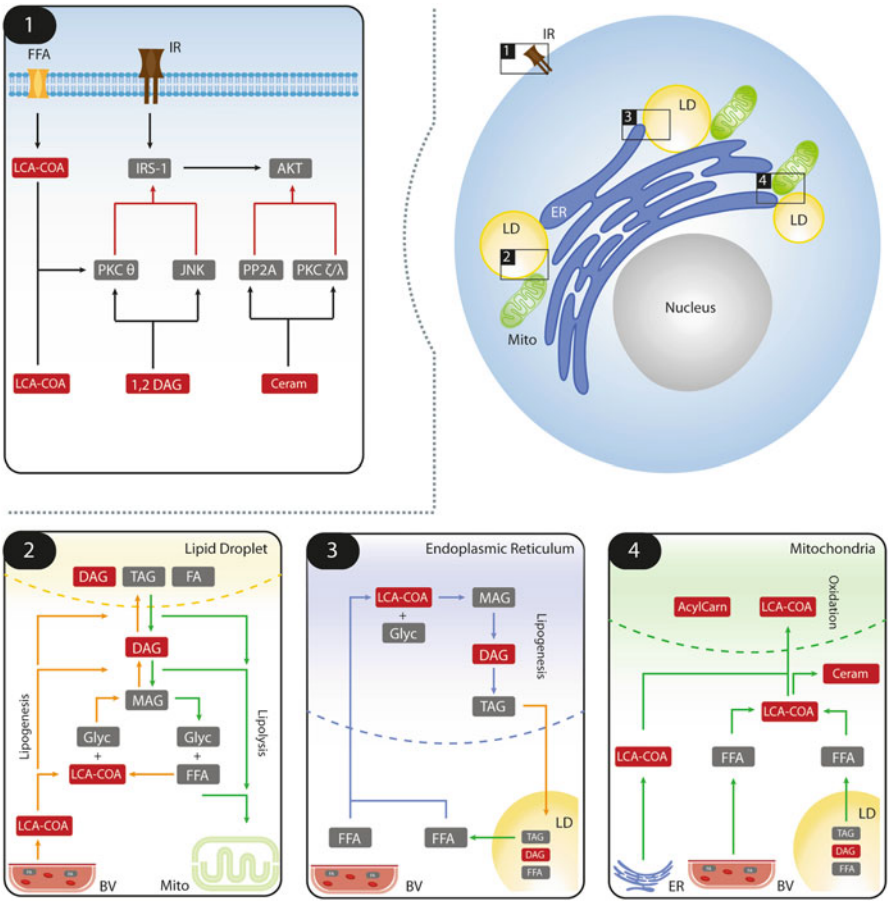

Fig. 1 Organization and location of the lipid intermediates at the subcellular level and their potential mechanisms for muscle insulin resistance. Panel 1 depicts a physiological perspective on how lipid intermediates may compromise proper insulin receptor substrate 1 (IRS-1) phosphorylation through the protein kinase C (PKC) θ and Jun N-terminal kinase (JNK) signaling pathways, and/or through the stimulation of protein phosphatase 2A (PP2A) and PKC ζ/λ downstream of the protein kinase B (AKT) interference. Panels 2, 3, and 4 provide a closer look to lipid intermediary metabolism within and between several organelles at the subcellular level (i.e., lipid droplets (LD), endoplasmic reticulum (ER), mitochondria (mito), and blood vessel (BV)). Boxes in red display lipid intermediates with possible links to insulin resistance; yellow arrows represent pathways leading to lipid storage; green arrows display pathways leading either to lipolysis or free fatty acid (FFA) oxidation at the mitochondria level; purple arrows are related with lipid synthesis at the endoplasmic reticulum level. Abbreviations: acylcarnitine, AcylCarn; long-chain acyl-CoAs, LCA-COA; diacylglycerols, DAG; glycerol, Glyc; insulin receptor, IR; monoglycerides, MAG; triglycerides, TAG.

The hypothesis of the novel DAG/protein kinase C (PKC) axis has forested a lot of interest by the scientific community as a possible link between lipid intermediates and the insulin resistance process. DAG is an intermediary in the synthesis and

breakdown of TG (Brouwers et al. 2016). It is a bioactive lipid involved in the signaling and activation of PKC, particularly PKC θ isoform in the muscle (Schmitz-Peiffer et al. 1997) and PKC ϵ isoform in the liver (Kumashiro et al. 2011). Although the evidence is not fully clear, most of the investigations in animal and human models suggest that the specific isoforms of PKC θ and ϵ can impair insulin action by phosphorylating the insulin receptor at the serine and threonine residue, respectively, thus, inhibiting IRS action (Petersen et al. 2016). DAG can exist as one of three stereoisomers (i.e., sn-1,2 sn-2,3, or sn-1,3); however, it is only the sn-1,2 that is capable of activating PKC θ and ϵ (Rando and Young 1984).

Finally, there is the ceramide hypothesis. Ceramides are bioactive lipids belonging to the sphingolipid family and are derived through either de novo synthesis from palmitate or re-acylation of sphingosine (i.e., salvage pathway) (Sokolowska and Blachnio-Zabielska 2019). Ceramides have been proposed to mediate skeletal muscle and liver insulin resistance through decreasing AKT activity by either stimulating the activation of PKC, specifically the PKC ζ and λ isoforms, and/or by stimulating protein phosphatase 2A (PP2A) (Sokolowska and Blachnio-Zabielska 2019), which results in decreased translocation of glucose transporter type 4 (GLUT4) to the plasma membrane and glucose uptake (Metcalfe et al. 2018).

Role of Exercise on Lipid Metabolism in T2DM

Alongside nutrition and medication, exercise remains a fundamental cornerstone in lifestyle interventions for individuals with T2DM to improve and control their lipid profile, as well as other risk factors. From a classical physiological approach, the delivery of FFA to the skeletal muscle during exercise is mainly supplied by the lipolysis of TGs stowed in the adipose tissue and the muscle. The main stimulus for lipid mobilization and the release of free fatty acids from the adipose tissue is mainly adrenergic, involving both circulating catecholamines and sympathetic enervation (Fritzen et al. 2020). If exercise is maintained for longer durations (>30–60 min), a significant increase in the concentrations of cortisol and growth hormone occurs, which stimulate the activity of lipolysis enzymes, such as adipose TG lipase and hormone-sensitive lipase. When it comes to T2DM, all these processes can be significantly impaired; however, it is still expected that FFA oxidation capacity can be enhanced through exercise and, thus, improve the overall lipid profile and metabolic flexibility in these individuals (Fritzen et al. 2020; Gemmink et al. 2020). In the previous sections, we have provided a background on how lipid metabolism is linked to insulin resistance. In the next section, we will address the role of acute and chronic exercise in countering lipotoxicity and improving overall insulin resistance through its action on the classic lipid biomarkers as well as on the lipid intermediates (Fig. 2). Finally, and before diving into the acute effects of exercise on the lipid profile, it is important to highlight that most of the experimental evidence arises from studies that used aerobic exercise with little information known on the impact of resistance training (RT) on the lipid intermediates in individuals with T2DM.

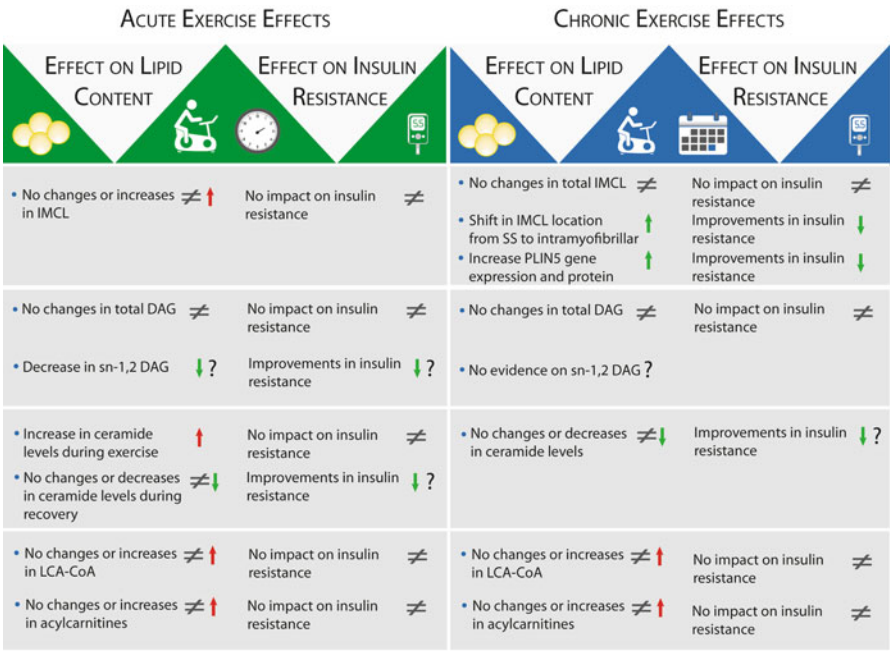

Fig. 2 Summary of the acute and chronic effects of exercise on lipid content and their impact on insulin resistance. Abbreviations: intramyocellular lipid, IMCL; subsarcolemmal, SS; diacylglycerols, DAG; long-chain acyl-CoAs, LCA-COA; perilipin protein 5, PLIN5

Acute Effects of Exercise on Muscle Lipid Content

Despite the proposed mechanisms and links between ectopic lipid accumulation, increased lipid intermediates, insulin resistance, and T2DM, high intramuscular lipid stores and elevated lipid intermediates have also been observed in endurance-trained athletes, an observation first identified by Goodpaster and colleagues and termed the "athlete paradox" (Goodpaster et al. 2001). However, unlike in individuals who have obesity or T2DM, insulin sensitivity of athletes is uncompromised and even enhanced despite the presence of IMCL (Gemmink et al. 2020). This paradox has provided a conceptual framework to understand the IMCL dynamics during acute and chronic exercise in both trained individuals and those with T2DM.

It has been hypothesized that the IMCL characteristics (i.e., size, proximity to mitochondria, expression of proteins regulating lipolysis) and dynamics (i.e., storage and breakdown of FFA), as well as mitochondrial oxidative capacity all play a major role in lipid droplet turnover, with higher turnover preventing lipid-induced toxicity (Bergman and Goodpaster 2020). The advent of stable isotope measurements and muscle biopsies obtained before and right after exercise has allowed a better understanding of the IMCL response to an acute bout of exercise, which is deeply dependent on the circulating FFA availability in both healthy and metabolically compromised individuals (Gemmink et al. 2020). There is, however, a significant difference when it comes to IMCL usage between trained and T2DM individuals,

with T2DM individuals relying little to none on the IMCL as a source of energy during an acute bout of exercise (Bergman et al. 2018). One possible explanation lies on the fact that individuals with T2DM have a dysfunctional adipose tissue with a higher rate of lipolytic activity and circulating levels of FFA, which could comprise the usage of IMCL as a viable energy source (Bergman et al. 2018). On the other end of the spectrum, athletes or exercise-trained individuals, have their IMCL stored in many smaller lipid droplets that are more proximally associated with a larger number of mitochondria, whereas individuals with T2DM tend to have fewer but larger lipid droplets that are found mainly around the cellular membrane of type II muscle fibers, with less lipid droplet-mitochondria interaction (Bergman and Goodpaster 2020). It has been hypothesized that mitochondria in the vicinity of the lipid droplets have privileged access to the FFA released by these depots and, hence, have a higher FFA oxidation rate, which will help maintain the ATP turnover during exercise (Gemmink et al. 2020). In T2DM individuals, the IMCL content during the recovery period following an acute bout of exercise has been reported to either increase or present no changes when compared to the baseline period (Bergman and Goodpaster 2020).

Acute Effects of Exercise on Muscle Lipid Intermediates

Considering the experimental evidence of the effects of acute aerobic exercise on DAG and sphingolipids, there is still limited data on these lipid intermediates. For instance, in the animal model and in humans with different metabolic statuses (i.e., trained athletes and T2DM individuals with obesity), a single bout of aerobic exercise did not impact the overall pool of DAG (Thrush et al. 2011; Bergman et al. 2018). Nonetheless, and as explained in the previous sections, more important than total DAG levels is the type of DAG stereoisomer present, with DAG sn-1,2 being involved in the activation of the PKC θ and ϵ and, hence, related with the insulin-resistant process (Rando and Young 1984). In this regard, IMCL lipolysis during exercise is responsible for the increased release of DAG sn-2,3 and sn-1,3. Therefore, insulin sensitivity may increase during exercise due to a decrease in DAG sn-1,2, since no changes have been observed in the overall pool of DAG, albeit further studies are needed to explore this potential mechanism (Bergman et al. 2018).

Given the pro-inflammatory nature of acute exercise, it has been observed that muscle levels of ceramide, among other lipid intermediates within the sphingolipids family, tend to increase temporarily. However, 2 h after a single bout of exercise ceramide values have been reported to decrease to values identical or less than those observed at baseline (Bergman et al. 2016). This observation has also been reported in the animal model and could partly explain the increase in insulin sensitivity following acute endurance exercise (Turinsky et al. 1990).

Finally, data on the impact of acute exercise on LCA-CoA and acylcarnitines remains controversial. For example, no changes or increases in LCA-CoA have been reported in animal studies following an acute bout of exercise (Li et al. 2015). Likewise, observations in humans have reported an increase or no change in acylcarnitines following an exercise session, regardless of the length of the chain

(Thyfault et al. 2010), which together mounts to a strong case that these polar lipids have no impact on the benefits in the insulin sensitivity that can be observed after an acute exercise session.

Chronic Effects of Exercise on Muscle Lipid Content

Similar to the athlete paradox, another contradictory observation arises from experimental studies addressing the long-term effects of exercise on IMCL content. When looking at the current body of literature in healthy trained individuals, some reports observed an increase in IMCL content, whereas no changes have been found in metabolically compromised patients, although both populations showed an increase in insulin sensitivity (Tarnopolsky et al. 2007; Toledo et al. 2008). It is plausible to assume that changes in insulin sensitivity following an exercise regimen do not rely on IMCL fluctuations, but instead rely on alterations in the lipid droplet characteristics. Experimental studies in both lean and obese individuals have reported a location shift of the lipid droplets residing within the muscle cell, which move from the subsarcolemmal to the intramyofibrillar space following long-term exercise (Tarnopolsky et al. 2007). These changes will impact the overall metabolic flexibility of the cell, since the intramyofibrillar lipid droplets are closer to the mitochondrial reticulum where they will approach a trained lipid droplet phenotype (Gemmink et al. 2020).

Another singularity within the "athlete paradox" concerns athletes/trained individuals having a greater abundance of lipid droplets coated with perilipin protein 5 (PLIN5c) compared to individuals with T2DM (Gemmink et al. 2020). The PLIN5 protein plays a major role alongside the lipases, such as the adipose TG lipase and hormone-sensitive lipase, in helping regulate the lipolysis from the lipid droplets in order for them to match the mitochondrial fat oxidation rate during exercise (Bosma et al. 2012). Chronic exercise above the 4-week duration threshold has been shown to increase PLIN5 gene expression and protein content in obese and T2DM patients (Shepherd et al. 2017). How these changes translate to an improved fat oxidation process in T2DM still warrants further investigation, since there are still mixed results between obese and T2DM individuals on the impact of chronic exercise on lipid droplet–mitochondrial tethering (Gemmink et al. 2020).

Chronic Effects of Exercise on Muscle Lipid Intermediates

Following the same trend of the previous subsection on the acute effects of exercise on lipid intermediates, there is still much to be understood on the chronic effects of exercise on DAG, sphingolipids, and polar lipids, especially on their isomers, species, and localization, and their relationship with changes in insulin resistance. This observation arises from experimental investigations showing that long-term endurance training increases DAG levels and insulin sensitivity in trained/athletic individuals, adding another layer to the conundrum of the "athlete paradox" (Amati et al. 2011). When looking at the individuals with obesity, the results of exercise interventions have shown no changes in overall DAG content (Ryan et al. 2020). Moreover, and as far as the sphingolipids family is concerned, chronic aerobic

exercise has been shown to decrease ceramide levels (Coen et al. 2015), with some studies showing no changes (Ryan et al. 2020). Finally, the LCA-CoA and acylcarnitines also appear to have no changes (Bruce et al. 2004) or even increase their content following chronic exercise (Ryan et al. 2020).

When gathering all the above information on the effects of chronic exercise on these lipid intermediates, it is clear that changes in insulin sensitivity are not dependent on favorable improvements of overall DAG, sphingolipids, and polar lipids content. Nonetheless, future studies addressing the impact of exercise on specific isomers, species, and location of these lipid biomarkers are paramount to fully understand their relationship with insulin sensitivity.

Effect of Exercise on Liver Lipid Content

Although the underlying mechanisms of the effects of exercise on IHL are poorly understood, exercise training has been proposed to lower IHL content by affecting one or more of the abovementioned pathways (Brouwers et al. 2016). First, exercise has a direct effect on lipoprotein lipase (LPL)-mediated TG removal from the circulation to skeletal muscle and as a consequence, there is lower TG uptake and accumulation by the liver and a decrease in hepatic VLDL secretion (Ussher 2014). These changes in LPL activity induced by exercise can partially explain the improved lipid profile observed in individuals with T2DM following training (Brouwers et al. 2016). Secondly, exercise promotes whole-body insulin sensitivity and hence insulin-stimulated suppression of lipolysis from adipose tissue and insulin-stimulated uptake of glucose and FFA by the muscle, all of which decrease the uptake of FFA by the liver (Brouwers et al. 2016; Gemmink et al. 2020). Beyond reduced uptake of FFA, exercise also reduces DNL by decreasing circulating insulin levels as well as glucose, which are key activators of DNL (Brouwers et al. 2016). Lastly, exercise can reduce IHL accumulation by increasing energy expenditure and inducing a negative energy balance. This in turn triggers the release and oxidation of FFA from IHL storage to fuel the increased energy demands of exercise (Ussher 2014). It has been suggested that this reduction in IHL due to increased FFA oxidation is one of the main mechanisms by which exercise improves NAFLD (Brouwers et al. 2016).

It is important to note that the reductions in IHL following exercise have been observed regardless of significant changes in body mass and total body fat. In fact, beyond changes in the quantity of IHL, exercise can also have an impact on its composition. For example, following just 7 days of exercise training, there was no change in the amount of IHL; however, the content on the IHL was significantly altered, such that it contained more polyunsaturated FFA (Haus et al. 2013). This finding is likely a result of the effect of exercise on suppressing DNL, where the principal FFA produced are saturated. Saturated FFA are known to negatively affect cellular functions, such as that of insulin signaling (Taskinen and Boren 2015). Thus, the qualitative changes in IHL following exercise in the absence of any quantitative changes can have positive implications on insulin sensitivity for individuals with T2DM.

Exercise Interventions and Classic Lipid Biomarkers

Many studies have highlighted the importance of exercise for improving metabolic health, including that of the classic lipid profile (Wood et al. 2019); however, fewer reviews have been conducted in the T2DM population, especially when trying to understand the impact of different types, intensities, and lengths of exercise interventions on the classic lipid biomarkers. In this section, we will address all the experimental evidence of exercise interventions with a RCT design that aimed to understand the impact of different types of training on the lipid profile. Mainly, we will focus on aerobic (e.g., with a special focus on moderate continuous training (MCT) and high-intensity training, either as interval or continuous), RT, and a combination of both aerobic and RT. Table 1 provides a summary of the results of recent RCT exercise interventions from 2010 forward on the classic lipid biomarkers in individuals with T2DM.

Overall, the majority of the evidence points to aerobic exercise of both vigorous or moderate intensity as being effective for improving the lipid profile (i.e., increasing HDL-C and decreasing total cholesterol (TC), LDL-C, and TG) in individuals with T2DM. Whether the benefits of aerobic training are superior when performed continuously or through repeated intervals remains unclear. Within the last decade, high-intensity interval training (HIIT) has become a popular training method and has been promoted as being more time-efficient, feasible, and effective than the traditional continuous endurance method. According to a recent systematic review and meta-analysis comparing the effects of HIIT and MCT on the lipid profile in both subclinical and clinical populations, HIIT protocols did not confer greater improvements in the lipid profile over MCT protocols (Wood et al. 2019). Nevertheless, it is difficult to generalize the effect of MCT versus HIIT on blood lipids, as much of the effect of these exercise regimens is likely influenced by the participant characteristics (age, sex, type of chronic disease, etc.) (Wood et al. 2019).

According to the most recent RCTs specific to individuals with T2DM, mixed results have also been reported. For instance, increases in HDL-C with HIIT and MCT have been observed compared to controls (Mitranun et al. 2014), whereas, no significant differences in the lipid profile have been reported following aerobic exercise training in other intervention studies, regardless of exercise intensity (Winding et al. 2018; Sabag et al. 2020). It is important to note that different HIIT protocols were implemented by most of these studies, with distinguished characteristics on the exercise-to-rest ratio as well as the intensity implemented. Thus, future investigations in individuals with T2DM are still warranted to draw conclusions on the role of interval versus continuous training on lipid biomarkers.

Considering the effects of RT on the lipid profile in individuals with T2DM, the majority of studies have reported no changes compared to controls, although a significant decreases in TC and TG compared to controls following a 12-week RT intervention has been reported (Dadrass et al. 2019). The inconsistency in these results could be due to differences in the baseline lipid profile of the included sample, since those with higher impaired values tend to have further exercise benefits.

Table 1 Outcomes of supervised exercise interventions on lipid biomarkers in individuals with T2DM according to different types of training.

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
Aerobic training					
Balducci et al. (2010)	Control: 20 (9/11) Exercise: 20 (8/12)	VCT: 70–80% VO_2max; treadmill and/or cycle ergometer	60 min/d, 2 d/week, 12 months	HDL-C	HDL-C increased compared to controls
Kadoglou et al. (2010)	Control: 21 (8/13) Exercise: 22 (8/14)	VCT: 50–80% VO_2ma; walking or jogging on treadmill, cycle ergometer, and calisthenics	45–60 min/day, 4 times/week, 12 months	TC, TG, HDL-C, LDL-C	TC, TG, and LDL-C decreased and HDL-C increased compared to controls
Sixt et al. (2010)	Control: 12 (8/4) Exercise: 11 (10/1)	VCT: 80% maximum heart rate, cycle ergometer	90 min/day, 5 days/week, 4 weeks, follow by 30 min/day, 5 days/week, plus 1 h/week supervised swimming or endurance training, 6 months; following 6 months unsupervised 5 days/week	TC, TG, HDL-C, LDL-C	TC and LDL-C decreased at 4 weeks but not after 6 months compared to controls
Belli et al. (2011)	Control: 10 (0/10) Exercise: 9 (0/9)	MCT: ventilatory threshold, outdoor walking	60 min/d, 3 d/week, 12 weeks	TC, TG, HDL-C, LDL-C	Exercise group increased HDL-C compared to controls
Jorge et al. (2011)	Control: 12 (4/8) Exercise: 12 (5/7)	VCT: lactate threshold; cycling	60 min/day, 3 days/week, 12 weeks	TC, TG, HDL-C	No significant difference compared to controls
de Oliveira et al. (2012)	Control: 12 (4/8) Exercise: 11 (5/6)	MCT: lactate threshold; cycling	50 min/d, 3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	No significant difference compared to controls
Arslan et al. (2014)	Control: 33 (14/19) Exercise: 31 (17/14)	VCT: 75% maximum HR, treadmill or bicycle	45 min/d, 3 d/week, 12 weeks	TC, TG, HDL-C, LDL-C	No significant difference compared to controls

(continued)

Table 1 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
Mitranun et al. (2014)	Control: 15 (5/10) MCT: 14 (5/9) HIIT: 14 (5/9)	MCT: 60% VO_2max HIIT: 6 × 1 min at 85% VO_2max, 4 min at 60% VO_2max	40 min/d, 3 d/week, 12 weeks	TC, TG, HDL-C, LDL-C	HDL-C increased in MCT and HIIT group vs. controls
Dehghan et al. (2016)	Control: 49 F Exercise: 49 F	MCT: 50–70% maximum heart rate, jogging	60 min/d, 3 d/week, 16 weeks	TC, TG, HDL-C, LDL-C	TC, TG, and LDL-C decreased and HDL-C increased compared to controls
Rahbar et al. (2017)	Control: 15 Exercise: 13	MCT: 50–70% maximum heart rate, treadmill	30 min/day, 3 times/week, 8 weeks	TC, TG, HDL-C, LDL-C, VLDL-C	No significant difference compared to controls
Saghebjoo et al. (2018)	Control: 10 F Exercise: 10 F	VCT: 75–85% maximum heart rate, jogging, running	From 15 min/day to 35 min/day, add 3–4 min per week, 3 times/week, 12 weeks	TG, HDL-C, LDL-C	TG, and LDL-C decreased and HDL-C increased compared to controls
Winding et al. (2018)	Control: 7 (5/2) MCT: 12 (7/5) HIIT: 13 (7/6)	MCT: cycling at 50% peak workload HIIT: cycling at 90% peak workload for 1 min followed by 1 min at 20% peak workload	MCT: 40 min/d, 3 days/week, 11 weeks HIIT: 20 min/d, 3 days/week, 11 weeks	TG, HDL-C, LDL-C	No significant difference compared to controls
Sabag et al. (2020)	Control: 11 (7/4) MCT: 12 (5/7) HIIT: 12 (7/5)	MCT: cycling at 60% VO_2max HIIT: 4 min cycling at 90% VO_2max (19 min total)	MCT: 40–55 min, 3 days/week, 12 weeks HIIT: minimum of 19 min, 3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C, FFA	No significant difference compared to controls
Resistance training					
Wycherley et al. (2010)	Control: 16 Exercise: 17	70–85% RM, 8–12 reps (leg press, knee extension, chest	45 min/d, 3 d/week, 16 weeks	TC, TG, HDL-C, LDL-C	No significant difference

(continued)

Table 1 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
		press, shoulder press, lat pull down, seated row, triceps press, and sit-ups)			compared to controls
Jorge et al. (2011)	Control: 12 (4/8) Exercise: 12 (5/7)	Leg press, bench press, lat pull down, seated rowing, shoulder press, abdominal curls, and knee curls	60 min/day, 3 days/week, 12 weeks	TC, TG, HDL-C	No significant difference compared to controls
Kadoglou et al. (2012)	Control: 24 (5/19) Exercise: 23 (7/16)	60–80% RM, 6–8 repetitions per exercise (seated leg press, knee extension, knee flexion, chest press, lat pull down, overhead press, biceps curl, and triceps extension)	45–60 min/day, 3 times/week, 3 months	TC, TG, HDL-C, LDL-C	No significant difference compared to controls
de Oliveira et al. (2012)	Control: 12 (4/8) Exercise: 10 (4/6)	50% RM, 4 sets of 8–12 reps (leg press, bench press, lat pull down, seated rowing, shoulder press, abdominal curls, knees curls)	3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	No significant difference compared to controls
Dadrass et al. (2019)	Control: 12 M Exercise: 12 M	First month 55% RM; second month 65% 1RM; third month 75% 1RM; 3 set of 10 repetitions (chest press, leg extension, leg curl, arm curl, push-up with knees against	50 min/day, 3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	TC and TG decreased compared to controls

(continued)

Table 1 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
		the floor, seated row, overhead pull down, overhead press, weighted sit-up and toe raise)			
Rech et al. (2019)	Control: 21 (10/11) Exercise: 17 (10/7)	2–3 sets of 12–10 reps (partial squat and bench stepping, unilateral leg press, unilateral knee extension, knee flexion, plantar flexion, bench press, low row, biceps curl, elbow extension, hip abduction and abdominal crunches)	3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	No significant difference compared to controls
Combined					
Balducci et al. (2010)	Control: 20 (9/11) Exercise: 22 (8/14)	VCT aerobic: 70–80% VO_2max; treadmill and/or cycle ergometer Resistance: 80% RM (chest press, lateral pull down, leg press, trunk flexion for the abdominals)	75 min/d, 2 days/week, 12 months	TC, TG, HDL-C, LDL-C	HDL increased and LDL decreased in exercise group compared to controls
Jorge et al. (2011)	Control:12 (4/8) Exercise: 12 (4/8)	VCT aerobic: lactate threshold; cycling Resistance: leg press, bench press, lat pull down, seated rowing, shoulder press, abdominal curls, and knee curls	60 min/day, 3 days/week, 12 weeks	TC, TG, HDL-C	No significant difference compared to controls

(continued)

Table 1 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
de Oliveira et al. (2012)	Control: 12 (4/8) Exercise: 10 (4/6)	MCT: 25 min cycle at lactate threshold Resistance: 2 sets of 8–12 RM	3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	No significant difference compared to controls
Kim et al. (2014)	Control: 17 (10/7) Exercise: 18 (9/9)	MCT aerobic: 50–70% maximum heart rate; brisk walking Resistance: 50% 10RM; 3 sets of 20 repetitions	70 min/day, 3 days/week, 12 weeks	TC, TG, HDL-C, LDL-C	TG decreased compared to the controls.
Annibalini et al. (2017)	Control: 8 M Exercise: 8 M	MCT aerobic: 40–65% heart rate reserve, walking on treadmill Resistance: 40–60% 1RM, 2–4 sets of 12–20 reps (horizontal leg press, lat pull down, chest press)	30–60 min/day, 3 days/week, 16 weeks	TC, HDL-C, LDL-C	TC decreased compared to control
Balducci et al. (2017)	Control: 150 M Exercise: 150 M	MCT aerobic: low to moderate intensity Resistance: not described	60 min/day, 2 days/week, 4 months	TC, TG, HDL-C, LDL-C	No significant difference from control
Magalhães et al. (2020)	Control: 27 (13/14) Exercise (HIIT): 25 (10/15) Exercise (MCT): 28 (15/13)	HIIT aerobic: 1 min of exercise at 90% of their heart rate reserve followed by 1 min resting at 40–60% of the heart rate reserve MCT aerobic: 40–60% of the heart rate reserve	Duration based on prescribed energy target, 3 days/week, 12 months	TC, TG, HDL-C, LDL-C	LDL, TC decreased with HIIT compared to controls

(continued)

Table 1 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Lipid outcomes	Results
		Resistance: 1 set of 10–12 RM (seated row, pull down, chest press, shoulder press, leg press, one leg lung, dead bug, and regular plank)			

VCT, vigorous continuous training; MCT, moderate continuous training; HIIT, high-intensity interval training; RM, maximum repetition; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very-low-density lipoprotein cholesterol; FFA, free fatty acid

Moreover, similar to the aerobic interventions, differences in the RT protocol used (i.e., intensity, frequency, duration) add to the difficulty in drawing conclusions on the effects of RT on the lipid profile.

Randomized controlled trials examining the effect of combined aerobic and RT on lipid biomarkers have had mixed results. For instance, when performing a 1-year exercise intervention, we (Magalhães et al. 2020) observed a favorable impact of HIIT combined with RT on TC and LDL-C when compared to the control group, whereas no changes were observed in the MCT combined with RT group. It is reasonable to deduce that the combination of resistance and aerobic exercises may provide additional benefits than either of them performed alone. However, the paucity of research of combined interventions with similar protocols makes it hard to come to any verdict.

Regardless of the exercise being aerobic (interval or continuous, moderate or high intensity), resistance, or a combination of the two, a major confounding factor when assessing the effects of exercise on the lipid profile is changes in body weight. It has been found that just small changes in body weight can have a considerable impact on insulin sensitivity at multiple organs (Petersen and Shulman 2018; Ryan et al. 2020). Moreover, and as previously explained, an increase in insulin sensitivity reduces circulating levels of glucose and lipids, via decreased lipolysis by adipose tissue, increased uptake by skeletal muscle, decreased fat influx and synthesis by the liver, and reduced liver VLDL secretion.

Overall, there is a clear link between exercise and an improved lipid profile, especially when it comes to aerobic exercise. If the intent is to answer the question, "what's the best exercise intensity and type to improve the lipid profile for individuals with T2DM," then more research is warranted, with the special caveat on standardizing the exercise intervention protocols.

Inflammatory Profile and T2DM

Introduction to Inflammation and T2DM

The inflammation process involves a complex cascade of events, where several cellular components are implicated to promote cell survival by warding off harmful infections derived from pathogenic bacteria, viruses, and parasites. Although the inflammatory profile is a key element in survival, there are circumstances in which it becomes unable to properly function, leading to maladaptive chronic states of low-grade inflammation. This low-grade inflammation is at the cornerstone of the pathophysiology involved in the development of T2DM. In the following sections ("The Relationship Between Obesity and Inflammation," and "The Relationship Between Inflammation and T2DM), we will examine the mechanisms linking several of the most studied inflammatory biomarkers in the exercise physiology field, with obesity and T2DM. We will then dive into how acute and chronic exercise may improve the adverse inflammatory profile inflicting individuals with T2DM, while also reviewing all the recent RCT on this topic (Section "Anti-inflammatory Effects of Exercise").

The Relationship Between Obesity and Inflammation

One of the underlying characteristics of people with T2DM is the overweight and obesity phenotype. At the epidemiological level, people with higher values of BMI, due to excessive body fat accumulation, have a higher relative risk of developing T2DM when compared with their lean peers (Petersen and Shulman 2018). Thus, it comes with no surprise that the divorce between energy expenditure and energy intake contributes to a positive energy balance, hence increasing body fat storage, which is one of the primary insults leading to chronic low-grade inflammation and T2DM (Pedersen 2009).

Energetic intake exceeding that of energy expenditure can lead to adipose tissue dysfunction, where local hypoxia and adipocyte apoptosis occur due to the increased homeostatic stress imposed by adipocyte hyperplasia and hypertrophy (Murano et al. 2008). As a consequence, chemotactic signals from strained and dead adipocytes are responsible for the recruitment of immune cells. As an example, the monocyte chemoattractant protein 1 (MCP-1) is an important cytokine that plays a major role in attracting other immune cells (e.g., macrophages, monocytes) to the extracellular space of the adipocytes, where these immune cells will be responsible for producing pro-inflammatory cytokines (Sartipy and Loskutoff 2003) (Fig. 3). Another possible pathway leading to adipose tissue macrophage recruitment is through the identification of adipocyte stress by the natural killer cells residing in visceral adipose tissue depots (Wensveen et al. 2015). These immune cells will in turn be involved in macrophage activation through the release of tumor necrosis factor-alpha (TNF-α) and interferon-y (Wensveen et al. 2015).

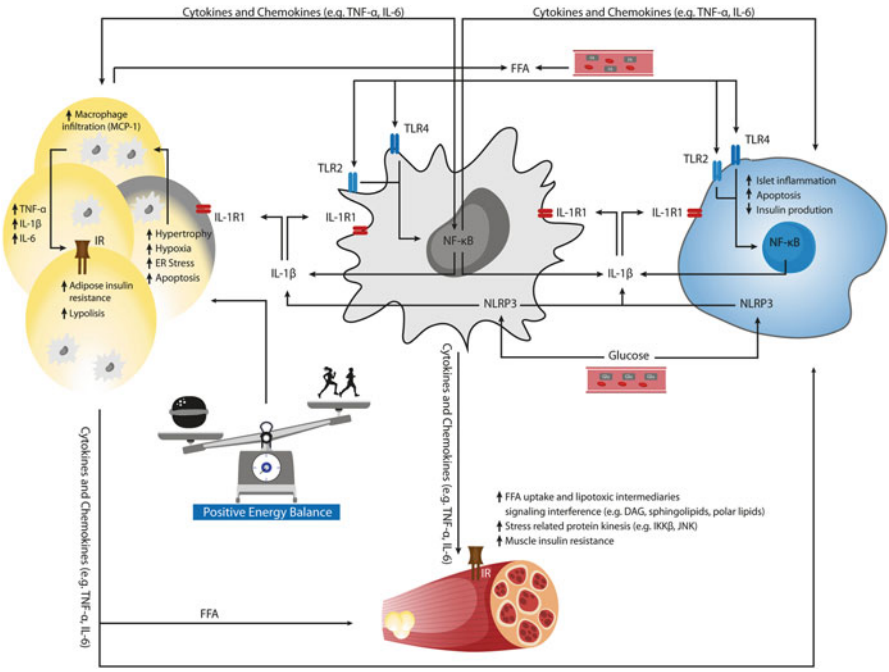

Fig. 3 An integrated physiological perspective on low-grade inflammation and its impact at the tissue and cellular level in T2DM. As a result of a positive energy balance due to low levels of physical activity and an increased energy intake, adipocytes become hypertrophic, placing them under significant stress that eventually can lead to cellular hypoxia and death. Consequently, the immune system will increase macrophage infiltration within the intercellular space of the adipose tissue, thereby increasing the release of cytokines/chemokines such as the tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), which will in turn affect adipose tissue insulin sensitivity. Local macrophages and other immune cells are responsible for creating a pro-inflammatory cycle perpetuating the release of more cytokines, which have autocrine effects as well as paracrine actions on other cells/organs such as the β-cells in the pancreas and the skeletal muscle. Fueling this inflammatory cycle, free fatty acids (FFA) arriving from the lipolytic adipose tissue or from the circulation bind to the toll-like receptors (TLR) 2 and 4 present on the macrophages and β-cells, triggering the downstream inflammatory nuclear factor kappa B (NF-κB) pathway. Moreover, elevated levels of circulating glucose are also involved in the release of IL-1β from macrophages and β-cells mediated by the NLR family pyrin domain containing 3 (NLRP3) sensor. At the pancreas, these cytokines are responsible for islet inflammation, leading to apoptosis and reduced insulin secretion. In the muscle, cytokines and FFA derived from the lipolytic adipose tissue are responsible for the muscle insulin resistance. Abbreviations: insulin receptor, IR; endoplasmic reticulum, ER; IL-1 receptor antagonist, IL-1R1; diacylglycerols, DAG; inhibitor kappa beta kinase beta, IKKβ; Jun N-terminal kinase, JNK.

An important link between obesity and inflammation also lies with the lipid profile. As previously mentioned in the section "The Relationship Between Obesity and Lipids," obese individuals with dysfunctional adipose tissue are prone to have impaired lipid profiles, characterized by elevated circulating levels of FFA, as well as

the accumulation of intercellular lipid intermediates (e.g., ceramides, DAG, acylcarnitines) (Petersen and Shulman 2018). Adipocyte-derived FFA and intercellular lipid intermediates can also contribute to low-grade chronic inflammation and macrophage activation mainly through toll-like receptors (TLR) and their nuclear factor kappa B (NF-κB) pathway (Donath et al. 2013). Beyond lipids, glucose can mediate the activation of the NLR family pyrin domain containing 3 (NLRP3)-dependent pathways, which directly affects the production of IL-1β (Donath et al. 2013) (Fig. 3). The activation and recruitment of these immune cells are responsible for inducing low-grade chronic inflammation. It has thus been proposed that the chronic inflammation associated with obesity-linked insulin resistance is initiated in adipose tissue.

The Relationship Between Inflammation and T2DM

To begin this discussion, it is important to clarify the role of the inflammatory process in T2DM: Is it the culprit behind the insulin resistance process, or is it rather an exacerbating factor? Several lines of experimental evidence have highlighted that although adipose tissue macrophage activation may induce a low-grade inflammatory process, this process is most likely an exacerbating factor of the obesity-associated insulin resistance, rather than a primary cause of the defect. As an example, investigations conducted using animal models have found that adipose tissue insulin resistance can be observed after 1-week of a high-fat diet (Cantley et al. 2013), but prominent macrophage infiltration and activation within the extracellular space of the adipocytes is only detectable after 12 weeks of high-fat feeding (Strissel et al. 2007). Nevertheless, regardless of whether it is a cause or exacerbating factor, low-grade chronic inflammation remains strictly linked to T2DM.

Within the inflammatory milieu, several pro-inflammatory cytokines have been identified as important contributors to the insulin resistance of adipose tissue and beta-cell dysfunction observed in T2DM. When looking at the recent literature, inflammatory biomarkers with predictive value for T2DM, such as the lipoprotein-associated phospholipase-A2, the trimethylamine-N-oxide, and the myeloperoxidase, have been associated with cardiovascular disease and have shown promising pharmacological therapeutics (Abdulhamied Alfaddagh et al. 2020). Nonetheless, there are no investigations addressing the impact of exercise on these biomarkers, thus, in the interest of keeping the scope of this chapter focused on exercise, we will address the chief among the inflammatory biomarkers, which include TNF-α, interleukin 1 beta (IL-1β), and interleukin 6 (IL-6).

TNF-α and T2DM

TNF-α was one of the first cytokines to be identified as a possible link between the adipose tissue low-grade inflammation and an insulin-resistant state (Hotamisligil et al. 1995). Considering all its members, the TNF superfamily displays pro-inflammatory activity, in which the inflammatory pathway depends in part on the activation of transcription factors, such as the NF-κB.

In vivo studies have shown that TNF-α increases lipolysis in humans without a concomitant increase in FFA oxidation at the muscle level, thus, leading to FFA incorporation into IMCL and lipid intermediaries, such as DAG, sphingolipids, and polar lipids as discussed in the previous section on lipids and T2DM. These lipid intermediates are responsible for the development of TNF-α-induced insulin resistance at the skeletal muscle. TNF-α can also impact the insulin-dependent pathway by inducing the serine phosphorylation of the insulin receptor substrate-1 (IRS-1), which blocks the downstream activation of phosphatidylinositol-3 kinase (PI3-kinase) and GLUT-4 translocation (Ozcan et al. 2004). This process is dependent on the activation of stress-related protein kinases such as the inhibitor kappa beta kinase beta (IKKβ), the Jun N-terminal kinase (JNK), and the NF-κB pathway (Ozcan et al. 2004).

This cytokine also displays an important role in insulin resistance by inducing β-cell dysfunction through the activation of the transcriptional factor NF-κB, which is responsible for increasing the inflammation and apoptosis of pancreatic islets, thus, leading to decreased insulin production (Bouzakri et al. 2011) (Fig. 3). Finally, TNF-α secretion as a result of low-grade chronic inflammation is related to increased atherogenic risk for the arterial wall (i.e., a major risk for the micro- and macrovascular complications associated with T2DM) by inducing the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and E-selectin (Couffinhal et al. 1993; Wyble et al. 1997).

IL-6 and T2DM

Circulating levels of IL-6 tend to be elevated in individuals with T2DM, with high concentrations being considered an independent predictor for the development of the disease (Akbari and Hassan-Zadeh 2018). However, the exact role of IL-6 in the pathogenesis of T2DM is somewhat controversial. This is mainly due to the pleiotropic behavior of IL-6, where it can have both beneficial and pathological effects on insulin sensitivity, insulin secretion, glucose homeostasis, as well as the inflammatory process depending on its origin (i.e., cytokine, adipokine, myokine), chronic or acute presence, cell target, and signaling mechanism (i.e., classical signaling through the membrane-bound IL-6 receptor (IL-6R) or trans-signaling through the soluble form of IL-6R (sIL-6R)) (Pedersen 2009; Han et al. 2020).

The link between IL-6 and T2DM is mainly due to its involvement in the signaling pathways that inhibit insulin signaling, which occurs in states of obesity-driven chronic inflammation (Donath et al. 2013). In adipocytes, chronically elevated levels of IL-6 increase the expression of the suppressor of cytokine signaling 3 (SOCS3), which reduces GLUT-4 mediated glucose uptake via phosphorylation of the insulin receptor β (IR-β), IRS-1, and PKB/Akt (Lagathu et al. 2003; Akbari and Hassan-Zadeh 2018). IL-6 can additionally reduce the expression of GLUT-4, IR-β, IRS-1, as well as peroxisome proliferator-activated receptor gamma (PPAR- γ), an insulin sensitizing factor (Rotter Sopasakis et al. 2004; Akbari and Hassan-Zadeh 2018). In the pancreatic islet cells, IL-6 tends to enhance β-cell apoptosis, which can contribute to decreased insulin secretion (Ellingsgaard et al. 2008). In the muscle cells, exposure to chronic levels of IL-6 seems to reduce insulin action through the

JNK, suppressor of cytokine signaling 3 (SOCS3), and protein tyrosine phosphatase 1B (PTP1B) pathways (Kim et al. 2004). In the liver, IL-6 has been shown to contribute to insulin resistance under both chronic and acute states (Akbari and Hassan-Zadeh 2018). IL-6 stimulates mTORC1, which activates signal transducer and activator of transcription (STAT), which in turn increases SOCS3 expression leading to the reduction in insulin signaling and glucose uptake (Kim et al. 2004).

Beyond disrupting insulin signaling, IL-6 contributes to the macro-vascular complications occurring in T2DM through the induction of C-reactive protein (CRP) gene expression in hepatocytes (Sproston and Ashworth 2018). CRP is considered an acute marker for inflammatory diseases and infections and is currently one of the few biomarkers that transitioned from a laboratory setting to being routinely used in the clinic (Sproston and Ashworth 2018). Given its general stability in repeated measurements over time, CRP is now considered an important biomarker for cardiovascular disease risk, adding incremental value to the traditional risk factors such as those of the lipid biomarkers (Emerging Risk Factors et al. 2012). Beyond being a marker, CRP is thought to be a mediator of the atherosclerotic process through promoting the expression of genes involved in monocyte adhesion, triggering LDL-C uptake by macrophages, and inhibiting endothelial nitric oxide synthase (Sproston and Ashworth 2018). CRP further promotes the inflammatory process through being involved in the production of other pro-inflammatory cytokines such as IL-1β and TNF-α (Sproston and Ashworth 2018).

IL-1β and T2DM

Like TNF-α and IL-6, IL-1β has also been found to play a major role in driving the local and systemic inflammation contributing to T2DM (Herder et al. 2015). However, like IL-6, the deleterious effects of IL-1β are dependent on its concentration and length of activation (Herder et al. 2015).

Under normal conditions, postprandial increases in glucose promote an acute rise in macrophages, which secrete IL-1β in a glucose-dependent manner (Dror et al. 2017). IL-1β subsequently binds to the IL-1 receptor on pancreatic β-cells and enhances the release of insulin by enhancing insulin granule docking at the plasma membrane (Hajmrle et al. 2016). Both insulin and IL-1β are responsible for promoting glucose uptake by muscles as well as macrophages (Dror et al. 2017). When in low concentrations and under conditions of acute exposure, IL-1β activates and delivers energy (i.e., glucose) to the innate immune system to aid in warding off unwanted microorganisms arising from the ingestion of food (Vandanmagsar et al. 2011).

Under states of obesity and excessive nutrient intake, macrophages resident in dysfunctional adipose tissue, particularly that found in visceral depots, synthesize and release IL-1β in response to elevated glucose levels and metabolic stress (Dinarello et al. 2010). In a paracrine fashion, IL-1β binds to the IL-1 receptor on adipose cells and stimulates the action of LPL, which in turn results in the release of FFA from circulating lipoproteins (Dinarello et al. 2010). IL-1β also reduces the transcriptional and posttranscriptional expression of the insulin receptor, impairing adipocyte insulin signaling (Jager et al. 2007). Circulating IL-1β, alongside FFA and

glucose, triggers the synthesis and release of IL-1β by the pancreatic β-cells, which, under chronically elevated levels, induces β-cell death (Donath et al. 2013). Furthermore, the β-cell death and IL-1β induced chemokines attract macrophages to the pancreas leading to islet inflammation and further β-cell destruction (Dinarello et al. 2010).

Anti-inflammatory Effects of Exercise

Since the beginning of the twenty-first century, the field of omics-based clinical discovery has seen a significant increase in research with the identification of more than 650 hormone-like substances, known as myokines, that are released from the muscle during exercise (Khan and Ghafoor 2019). This research has fostered new therapeutic opportunities for several pathophysiological conditions, as well as uncertainty, warranting further investigation to understand how these myokines regulate the muscle-organ crosstalk. Now the muscle is no longer seen only as a tissue with contractile functions responsible for movement, heat, and strength production, but instead it is capable of impacting several biomolecular functions through its secretome, reaching diverse tissues such as the brain, liver, bone, gut, skin, pancreas, adipose tissue, as well as other cells such as those of the immune system (Pedersen 2006). Given the impact of the muscle secretome in ensuring whole-body homeostasis, including preventing low-grade chronic inflammation, these next sections will address some of the anti-inflammatory mechanisms mediated by myokines as well as other potential hormonal factors of acute and chronic exercise.

Acute Effects of Exercise and Anti-inflammatory Mechanisms

Myokines and their extensive portfolio of hormone-like substances, the IL-6 stands out as the prototype adipo-myokine with a considerable amount of research performed to explore its anti-inflammatory effects following a single bout of exercise (Pedersen 2017). As mentioned previously, IL-6 can have both pro- and anti-inflammatory actions, depending on several factors, with one being dependent on the cells responsible for its production (i.e., adipocytes (adipokine), macrophages (cytokine), or muscle (myokine)), with IL-6 secreted by muscle having a more anti-inflammatory profile. Moreover, the inflammatory/anti-inflammatory effects of IL-6 depend on how the signaling between the protein and its receptor unfold (Han et al. 2020). On this topic, Han and colleagues described that a switch of IL-6 signaling from a canonical mode to a noncanonical trans-signaling mode occurs due to the increased expression of the ADAM10/17 metalloprotease, which enhances trans-signaling via the soluble IL-6 receptor α (Han et al. 2020). This trans-signaling by IL-6 is known to promote adipose tissue M1 macrophage recruitment and a more inflammatory phenotype.

Looking at exercise, IL-6 can increase its circulating levels several fold, depending on factors such as the duration, intensity, and type of exercise, as well as nutritional intake of carbohydrates and pre-exercise glycogen content (Pedersen

2006; Pedersen 2017). For instance, following an acute bout of 30 min running at 75% of VO_{2max}, the circulating levels of IL-6 were increased by five-fold, whereas after a marathon, the values were up by 100-fold (Ostrowski et al. 1999). These differences between a shorter duration of exercise to a prolonged bout, as observed in long-distance running (e.g., marathons), highlight the importance of the duration of exercise as a considerable factor impacting the magnitude of changes in IL-6 production. A possible explanation for these observations lies on the glycogen reserves of the muscle following exercise. Both IL-6 mRNA and protein content increase drastically after long-duration exercise when glycogen content tends to decrease significantly (Pedersen 2006). The levels of acute IL-6 production following longer periods of exercise training are more pronounced in untrained sedentary individuals where glycogen reserves are lower when compared with their athletic peers (Pedersen 2017). Moreover, chronic adaptations to exercise, such as increased glycogen content, lead to reduced levels of IL-6 production when the same relative intensity of exercise is considered between untrained and trained individuals (Pedersen 2017). In fact, a nutritional intake of carbohydrates during exercise will impact the circulating levels of glucose and preserve glycogen reserves, hence blunting the circulating levels of IL-6 (Pedersen and Febbraio 2008).

Alongside the duration, intensity and type of exercise also play a major role in the levels of circulating IL-6, since the amount of IL-6 production is directly correlated with the amount of muscle mass recruited during the exercise (Pedersen 2017). Both concentric and eccentric exercises are known to increase the circulating levels of IL-6, with eccentric exercise increasing IL-6 release to a bigger extent (Bruunsgaard et al. 1997). These observations follow the hypothesis that IL-6 levels may be related with exercise muscle damage; however, non-damaging exercises with a more noticeable anti-inflammatory nature can also increase IL-6 values (Bruunsgaard et al. 1997).

Given its pronounced expression during exercise, IL-6 is one of the most important myokines in regulating the anti-inflammatory response of exercise. IL-6 release is responsible for inducing downstream increases of IL-1 receptor antagonist (IL-1ra) and IL-10 by blood mononuclear cells, all of which have anti-inflammatory properties (Dinarello 1994; Steensberg et al. 2003). After exercise, IL-1ra will inhibit IL-1β signal transduction, whereas IL-10 will be responsible for inhibiting the synthesis of pro-inflammatory cytokines such as TNF-α (Dinarello 1994; Opp et al. 1995). Beyond the acute effects on IL-6, exercise is also responsible for several hormonal changes that have a direct impact on the inflammatory profile. For instance, catecholamines and cortisol production can be responsible for some of the anti-inflammatory effects of exercise by blunting the release of TNF-α and by boosting the circulating levels of neutrophils, respectively (van der Poll et al. 1996).

Chronic Effects of Exercise and Anti-inflammatory Mechanisms

By increasing the frequency of exercise sessions, individuals with low-grade chronic inflammation (i.e., such as those with T2DM) will benefit from the stacked anti-inflammatory effects of exercise-induced IL-6 production (Pedersen 2006). Trained individuals have lower plasma IL-6 concentrations at rest and after exercise as a

consequence of training adaptations (Pedersen 2017). There are other mechanisms through which exercise can exert its chronic effects and regulate several biomarkers related to the inflammatory milieu. Some of the most important ones include the direct and indirect effects of exercise on reducing visceral adipose tissue, either by promoting a negative energy balance or through the mediating effects of adipokines and myokines (Graf and Ferrari 2019). By preventing/reducing the accumulation of adipose tissue, regular exercise will have a favorable impact on adipose tissue-derived pro-inflammatory cytokines such as the MCP-1, which in turn will reduce the number of immune cells infiltrated within the adipocytes, and consequently lower the circulating levels of IL-1β and TNF-α (Troseid et al. 2004). There are also other myokines along with IL-6 that are upregulated during exercise with important roles in the lipid metabolism, such as the IL-15 and brain-derived neurotrophic factor (BDNF) (Graf and Ferrari 2019). Strong physiological evidence suggests that IL-15 can regulate lipid deposition in preadipocytes and decrease the amount of white adipose tissue, whereas IL-6 and BDNF are responsible for increasing fat oxidation via the AMP-activated protein kinase (AMPK) pathway (Graf and Ferrari 2019). Moreover, IL-15, IL-6, myostatin, IL-7, IL-4, and the leukemia inhibitory factor (LIF) are all involved in skeletal muscle growth and maintenance, providing an essential increase in the amount of active metabolic tissue in the body, which will help prevent the accumulation of visceral adipose tissue (Pedersen 2017).

Another important mechanism where regular exercise can impact the inflammatory profile lies with the changes in the proportion of pro- versus anti-inflammatory monocytes in the blood, with the pro-inflammatory monocytes (i.e., $CD14^{low}CD16^{+}$), which have 2.5 times more cell surface TLR4, being reduced following an exercise program (Skinner et al. 2005; Gleeson et al. 2011). Given that glucocorticoid therapy has been shown to blunt the circulating levels of $CD14^{low}CD16^{+}$ monocytes, the effect of prolonged exercise on the number of $CD14^{low}CD16^{+}$ may be mediated through its effects on cortisol release (Fingerle-Rowson et al. 1998; Gleeson et al. 2011). Additionally, exercise has also been linked to a reduction in the expression of TLR1, TLR2, and TLR4 on monocytes, and hence, impacts the downstream inflammatory pathways by lowering the inflammatory cytokine production (Stewart et al. 2005). Overall, exercise is responsible for decreasing low-grade chronic inflammation through multiple mechanisms and if maintained on the medium to long term, it can improve whole-body insulin sensitivity in people with T2DM (Fig. 4).

Exercise Interventions and Inflammatory Biomarkers

In this section, we will address all of the most recent experimental evidence on exercise interventions with a RCT design that aimed to understand the impact of different types of training on the inflammatory profile. Mainly, we will focus on aerobic (e.g., MCT and high-intensity training, either interval or a continuous one), RT, and a combination of both aerobic and RT (Table 2).

When it comes to aerobic exercise, consistent decreases in IL-6, TNF-α, and CRP were observed following MCT protocols compared to controls when the intervention length was greater than 12 weeks (Abd El-Kader and Saiem Al-Dahr 2016; Dehghan

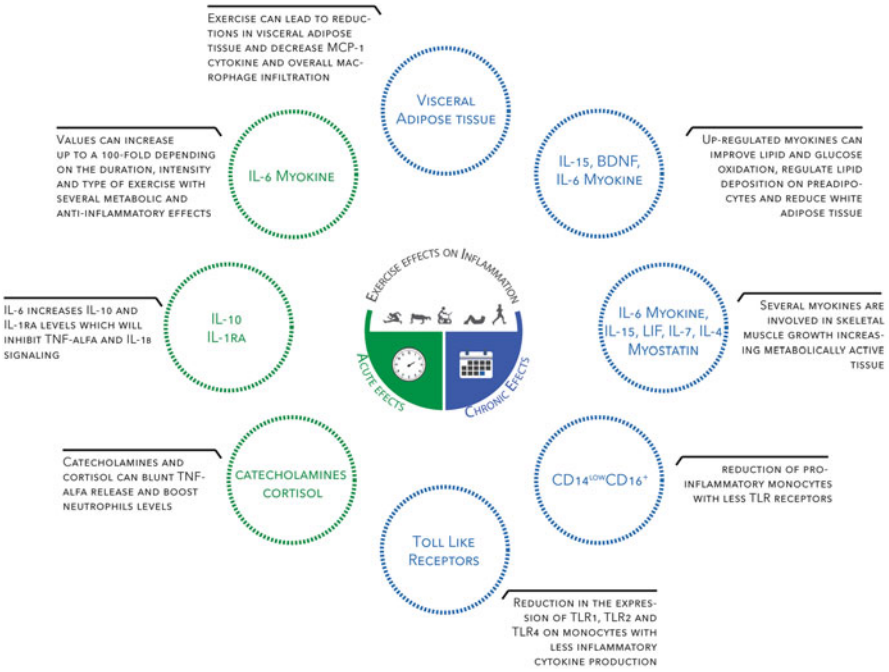

Fig. 4 Summary of the acute and chronic effects of exercise on the inflammatory profile. Abbreviations: tumor necrosis factor-alpha, TNF-α; interleukin-1 beta IL-1β; IL-1 receptor antagonist, IL-1R1; interleukin-6, IL-6; interleukin-4, IL-4; interleukin-15, IL-15; interleukin-7, IL-7; leukemia inhibitory factor, LIF; toll-like receptor, TLR; monocyte chemoattractant protein 1, MCP-1

et al. 2016). Similar to MCT aerobic exercise, vigorous continuous training also led to decreases in inflammatory (CRP and IL-18) and increases in anti-inflammatory (IL-10) markers (Kadoglou et al. 2007; Kadoglou et al. 2010; Saghebjoo et al. 2018).

Less investigation has been performed when it comes to assessing the effects of RT on inflammatory biomarkers, with a lack of consistent results being reported across studies. For instance, RT studies performed 3 times per week, with a duration of between 12 and 16 weeks, have found no changes in CRP when compared to controls (Wycherley et al. 2010; Kadoglou et al. 2012; Dadrass et al. 2019; Rech et al. 2019), although some did report favorable changes in TNF-α and IL-6 (Dadrass et al. 2019). Despite similarities in duration and frequency, most of the previous RT studies have employed different RT protocols (i.e., training intensity and number of reps and sets), making it difficult to compare results between studies. Also, some of the studies did not fully describe the training protocol used (i.e., intensity, frequency, duration), which further adds to the difficulty of study comparison and deriving any conclusion on the effects of RT alone on inflammatory biomarkers.

With combined training, results are mixed as well. For instance, we found that IL-6 decreased following either MCT and RT or HIIT and RT (Magalhães et al. 2020). Likewise, others have observed improvements in IL-6 with MCT and RT

Table 2 Outcomes of supervised exercise interventions on inflammatory biomarkers in individuals with T2DM according to different types of training

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
Aerobic					
Balducci et al. (2010)	Control: 20 (9/11) Exercise: 20 (8/12)	VCT: 70–80% VO_2max; treadmill and/or cycle ergometer	60 min/d, 2 d/week, 12 months	CRP, TNF-α, IL-6, IL-1β, IL-4, IL-10, IFN-g	No significant difference from control
Kadoglou et al. (2010)	Control: 21 (8/13) Exercise: 22 (8/14)	VCT: 50–80% VO_2max; walking or jogging on treadmill, cycle ergometer, and calisthenics	45–60 min/day, 4 times/week, 12 months	hsCRP, IL-10, IL-18	hsCRP decreased compared to controls
Sixt et al. (2010)	Control: 12 (8/4) Exercise: 11 (10/1)	VCT: 80% maximum heart rate, cycle ergometer	90 min/day, 5 days/week, 4 weeks, follow by 30 min/day, 5 days/week, plus 1 h/week supervised swimming or endurance training, 6 months; following 6 months unsupervised 5 days/week	hsCRP	hsCRP significantly decreased at 4 weeks but not after 6 months compared to controls
Arslan et al. (2014)	Control: 33 (14/19) Exercise: 31 (17/14)	VCT: 75% maximum heart rate, treadmill or bicycle	45 min/d, 3 d/week, 12 weeks	TNF-α	No significant difference from control
Abd El-Kader (2016)	Control: 40 (21/19) Exercise: 40 (23/17)	MCT: 70% maximum heart rate; row and cycle ergometer	30 min/day, 3 times/week, 12 weeks	TNF-α, IL-6, CRP	TNF-α, IL-6, and CRP decreased in exercise group compared to controls
Dehghan et al. (2016)	Control: 49 F Exercise: 49 F	MCT: 50–70% maximum heart rate, jogging	60 min/d, 3 d/week, 16 weeks	CRP, NF-κB1	CRP and NF-κB1 decreased and significantly different than control

(continued)

Table 2 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
Rahbar et al. (2017)	Control: 15 Exercise: 13	MCT: 50–70% maximum heart rate, treadmill	30 min/day, 3 times/week, 8 weeks	CRP	No significant difference from controls
Karimi et al. (2017), Shakil-Ur-Rehman (2017)	Control: 51 (19/32) Exercise: 51 (36/15)	Intensity not stated; 0 degree incline for 5 weeks, increase 3 degrees per 5 weeks; treadmill	10 min/day for the first 5 weeks (3 times/week), increase 30 min per 5 weeks; 25 weeks	IL-6	IL-6 decreased compared to controls
Saghebjoo et al. (2018)	Control: 10 F Exercise: 10 F	VCT: 75–85% maximum heart rate, jogging, running	From 15 min/day to 35 min/day, add 3–4 min per week, 3 times/week, 12 weeks	hsCRP, TNF-α	hsCRP and TNF-α decreased compared to controls
Resistance					
Wycherley et al. (2010)	Control: 16 M Exercise: 17 M	70–85% 1RM; 2 sets of 8–12 repetitions (leg press, knee extension, chest press, shoulder press, lat pull down, seated row, triceps press, and sit-ups)	45 min/day, 3 days/week, 16 weeks	CRP	No significant difference from control
Kadoglou et al. (2012)	Control: 24 (5/19) Exercise: 23 (7/16)	60–80% 1RM, 6–8 repetitions per exercise (seated leg press, knee extension, knee flexion, chest press, lat pull down, overhead press, biceps curl, and triceps extension)	45–60 min/day, 3 times/week, 3 months	hsCRP	No significant difference from controls

(continued)

Table 2 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
Dadrass et al. (2019)	Control: 12 M Exercise: 12 M	First month 55% 1RM; second month 65% 1RM; third month 75% 1RM; 3 set of 10 repetitions (chest press, leg extension, leg curl, arm curl, push-up with knees against the floor, seated row, overhead pull down, overhead press, weighted sit-up and toe raise)	50 min/day, 3 days/week, 12 weeks	CRP, TNF-α, IL-6	TNF-α and IL-6 decreased compared to controls
Rech et al. (2019)	Control: 21 (10/11) Exercise: 17 (10/7)	2–3 sets of 12–10 reps (partial squat and bench stepping, unilateral leg press, unilateral knee extension, knee flexion, plantar flexion, bench press, low row, biceps curl, elbow extension, hip abduction and abdominal crunches)	3 days/wee, 12 weeks	TNF-α, CRP, IL-6, IL-10, IL-1β	No significant difference from controls
Combined					
Balducci et al. (2010)	Control: 20 (9/11) Exercise: 22 (8/14)	VCT aerobic: 70–80% VO_2max; treadmill and/or cycle ergometer	(40 aerobic+20 resistance) min/day, 2 days/week, 12 months	hsCRP, TNF-α, IL-6, IL-1β, IL-4, IL-10	hsCRP and IL-6 significantly decreased compared to controls

(continued)

Table 2 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
		Resistance: 80% 1RM (thrust movement, chest press, lateral pull down, leg press, trunk flexion for the abdominals			
Kim et al. (2014)	Control: 17 (10/7) Exercise: 18 (9/9)	MCT aerobic: 50–70% maximum heart rate; brisk walking Resistance: 50% 10RM; 3 sets of 20 repetitions	70 min/day, 3 days/week, 12 weeks	hsCRP	No significant difference from controls
Annibalini et al. (2017)	Control: 8 M Exercise: 8 M	MCT aerobic: 40–65% heart rate reserve, walking on treadmill Resistance: 40–60% 1RM, 2–4 sets of 12–20 reps (horizontal leg press, lat pull down, chest press)	30–60 min/day, 3 days/week, 16 weeks	hsCRP, TNF-α, IL-6, MCP-1	TNF-α, IL-6, and MCP-1 decreased compared to controls
Balducci et al. (2017)	Control: 150 M Exercise: 150 M	MCT aerobic: low to moderate intensity Resistance: not described	60 min/day, 2 days/week, 4 months	hsCRP	No significant difference from controls
Banitalebi et al. (2019)	Control: 14 F Exercise: 14 F	MCT aerobic: 60–70% maximum heart rate; treadmill or cycle ergometer Resistance: 1 set of 15 reps, for the first 2 weeks,	3 days/week, 10 weeks	IL-6, IL-15	IL-6 decreased compared to controls

(continued)

Table 2 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
		increased to 2–3 sets of 12–10 reps with 12–10 reps/set between weeks 3 and 10; (bilateral leg press, lateral pull down, bench press, bilateral biceps curl, and bilateral triceps push down)			
Zaidi et al. (2019)	Control: 68 (54/14) Exercise: 69 (61/8)	Aerobic: interval training with 5–15 min at rate of perceived exertion (RPE) $\geq$ 15 and remaining exercise time at RPE = 12–14 Resistance: 10–15 reps (chest, biceps, shoulder, triceps, back and front)	60 min/d, 3 days/week, 12 months	IL-18 (circulating) IL-18 (gene expression leukocyte) IL-18 (gene expression adipose) NLRP3 (gene expression leukocyte) Caspase-1 (gene expression leukocyte)	No significant difference from controls
Magalhães et al. (2020)	Control: 27 (13/14) Exercise (HIIT): 25 (10/15) Exercise (MCT): 28 (15/13)	HIIT aerobic: 1 min of exercise at 90% of their HRR followed by 1 min resting at 40–60% of the heart rate reserve MCT aerobic: 40–60% of the heart rate reserve Resistance: 1 set of 10–12 RM (seated	Duration based on prescribed energy target, 3 days/week, 12 months	sCD163, TNF-α, IL-6, CRP	IL-6 decreased compared to controls; no significant differences from controls for sCD163, TNF-α, and CRP

(continued)

Table 2 (continued)

Study	Sample size (M/F)	Intervention intensity	Duration	Inflammatory outcomes	Results
		row, pull down, chest press, shoulder press, leg press, one leg lung, dead bug and regular plank)			

VCT, vigorous continuous training; MCT, moderate continuous training; RM, maximum repetition; CRP, C-reactive protein; hsCRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-10, interleukin 10; IL-15, interleukin 15; IL-18, interleukin 18; IFN-γ, interferon gamma; NF-κB1, nuclear factor-kappa B; MCP-1, monocyte chemoattractant protein-1; sCD163, soluble CD163; NLRP3, NLR family pyrin domain containing 3

(Annibalini et al. 2017). However, when combining RT with vigorous continuous training, the Italian Diabetes and Exercise Study found no changes in IL-6, but did observe decreases in CRP (Balducci et al. 2010).

Although exercise does improve inflammatory biomarkers, the type, intensity, duration, and frequency of exercise to achieve these improvements is still not clear. It is important to highlight that many of the anti-inflammatory benefits of exercise are mediated by changes in body weight and body composition. For example, no changes were found in TNF-α and CRP after 6 months of aerobic training 2 times per week in individuals with T2DM without weight loss (Zoppini et al. 2006). Alternatively, individuals with T2DM who performed aerobic training for 12 weeks, lost body weight while also decreasing their levels of TNF-α and CRP compared to non-exercising controls (Abd El-Kader and Saiem Al-Dahr 2016). Beyond total body weight loss, alterations in body composition with decreasing proportions of body fat can also be involved in the exercise-induced alterations on inflammatory biomarkers. For instance, despite no changes in overall body weight, a greater effect size for improvements in inflammatory biomarkers following 1-year of exercise training was observed in the group of individuals with T2DM who had decreases in abdominal and total body fat mass compared to the group without significant improvements (Magalhães et al. 2020).

Applications to Other Diseases or Conditions

Beyond T2DM, lipid and inflammatory profiles also play a major role in the pathogenesis of other diseases such as that of cardiovascular disease. For instance, the atherosclerotic process begins by lipoprotein-driven inflammation, which involves LDL-C penetrating into the intima tunica of the artery walls, where it undergoes oxidation and/or enzymatic modifications, triggering an immune

response with the activation and recruitment of monocytes/macrophages and T cells. Monocytes adhere to activated endothelial cells and infiltrate the vessel wall, becoming lesional macrophages that engulf the oxidized and modified LDL-C. These lipid-laden macrophages, otherwise known as foam cells, as well as other resident cells, eventually die further propagating the inflammatory process. Cytokines secreted by damaged endothelial cells promote smooth muscle cell proliferation and migration from the tunica media into the intima, forming of a fibrous capsule covering the foam cells and necrotic cell debris. Unstable fibrous plaque eventually rupture, leading to thrombosis and occlusions to the artery (Seidman et al. 2014).

Similar to T2DM, exercise induced alterations to the lipid profile and inflammatory biomarkers can aid in the treatment and management of cardiovascular disease. As discussed in this chapter, regular exercise can reduce the proportion of pro- versus anti-inflammatory monocytes in the blood by decreasing their expression of toll-like receptors (TLR) that instigate downstream pro-inflammatory cytokine production (Skinner et al. 2005; Stewart et al. 2005; Gleeson et al. 2011). Given that pro-inflammatory monocytes (i.e., $CD14^{low}CD16^{+}$) have a stronger propensity to adhere to activated arterial endothelial cells and become classically activated M1 macrophages that secrete pro-inflammatory cytokines, exercise can play a pivotal role in detaining and reversing the continued pro-inflammatory cascade that leads to further arterial damage and plaque development (Troseid et al. 2004; Colin et al. 2014). Moreover, as previously mentioned, exercise can increase lipoprotein lipase activity and FFA oxidation capacity, thus regulating the circulating lipoprotein profile, including that of LDL-C, and improving the overall risk of cardiovascular disease.

Walking alongside cardiovascular diseases, there are other chronic diseases, such as cancer and neurodegenerative diseases, that fall under the prophylactic umbrella of physical exercise. When looking at the modern challenges of the human species, the role of exercise has a therapeutic tool for chronic disease prevention and improving overall quality of life is now more important than ever. In fact, there are several investigations currently undergoing in both humans and in the animal model, exploring the molecular transducers and overall mechanisms underlying the benefits of a physically active lifestyle on chronic diseases, which will ultimately improve our understating of this intricate relationship.

Conclusion

With this chapter, we provided an overview on the treatment regimes in T2DM, specifically that of exercise, and its impact on the inflammatory and lipid profile, which are two of the most rapidly growing fields of research. Despite the well-known benefits of exercise for all population segments, and especially for those with T2DM, its widespread use in a clinical and day-to-day setting is far from ideal. In fact, when considering the latest challenges imposed by our modern society, it is clear that lifestyle interventions, where exercise is used as a cornerstone strategy, are

paramount for the control and treatment of T2DM. It is with this intent that this chapter provides the latest developments on the relationship between exercise and lipid biomarkers, such as lipid intermediates, and muscle and liver ectopic fat, as well as the most studied inflammatory biomarkers (i.e., IL-6, IL-1β, and TNF-α). Given that in the last decade we witness a rise in the amount of new health-related biomarkers, as a consequence of the advent of mass spectrometry-based proteomics, it is expected that we will continue to observe the same trend in the future. Therefore, RCT-designed interventions should take advantage and study the effects of exercise on these biomarkers, in order to explore new therapeutic targets and solutions for the treatment and control of T2DM.

Mini-Dictionary of Terms

Adipo-myokine. Small peptide secreted by both the adipose tissue or muscle cells that facilitate the interaction and communication between cells either through autocrine (self), paracrine (nearby cells), and/or endocrine (distant cells) action (e.g., IL-6).

Athlete paradox. Phenomenon observed in endurance-trained athletes, who have high insulin sensitivity and oxidative capacity despite elevated levels of intramyocellular lipids and lipid intermediates (e.g., DAG, ceramides).

Cytokine. Small peptide secreted by immune cells that facilitate the interaction and communication between cells either through autocrine (self), paracrine (nearby cells), and/or endocrine (distant cells) action (e.g., tumor necrosis factor alpha, interleukin-1 beta, interleukin-6).

Lipid intermediates. Bioactive lipid species or metabolites formed during free fatty acid (FFA) metabolism, which are thought to mediate lipid-induced insulin resistance (e.g., diacylglycerol (DAG), sphingolipids, long-chain acyl-CoAs, acylcarnitines).

Lipoprotein. Macromolecular particles originating from the liver (i.e., VLDL) and intestine (i.e., chylomicron) that facilitate the transport of cholesterol and triglycerides through the blood to other tissues of the body.

Metabolic flexibility. Ability of cells to quickly respond or adapt their sources of fuel for oxidation to conditional changes in metabolic or energy demands.

Myokine. Small peptide secreted by skeletal muscle in response to muscular contractions that communicate between muscle and other organs via autocrine (self), paracrine (nearby cells), and/or endocrine (distant cells) action (e.g., interleukin-6, interleukin-4, interleukin-7, interleukin 15, leukemia inhibitory factor, brain-derived neurotrophic factor).

Nonalcoholic fatty liver disease (NAFLD). Ectopic accumulation of fat in the liver, which is a common comorbidity coexisting in ~70% of individuals with T2DM

Type 2 diabetes mellitus. A noncommunicable chronic disease characterized by resistance to the peripheral actions of insulin as well as impairments in insulin secretion by the pancreas.

Key Facts on Exercise and the Lipid Profile

- Exercise optimizes mitochondrial capacity to switch between oxidative fuels in response to nutritional and physiological cues, thus enhancing overall metabolic flexibility.
- Exercise promotes intramuscular lipid (IMCL) usage through increasing the rate of FFA oxidation and by the rearrangement of intramuscular lipid droplets from the subsarcolemmal to the intramyofibrillar space to be closer to the mitochondria.
- Exercise increases diacylglycerol (DAG) sn-2,3 and sn-1,3 via the increase utilization of IMCL, hence, decreasing DAG sn-1,2 with possible implications in increased insulin sensitivity.
- Exercise increases lipoprotein lipase (LPL) activity and, thus, TG removal from the circulation to skeletal muscle TG.
- Exercise promotes whole body insulin sensitivity resulting in insulin-stimulated suppression of lipolysis in adipose tissue, insulin-stimulated uptake of glucose and fatty acids by the muscle, and the reduction of de novo lipogenesis by the liver.

Key Facts on Exercise and Inflammation

- Acute exercise promotes the production and secretion of IL-6 from skeletal muscle cells, which has anti-inflammatory effects through stimulating IL-10 production and IL-1 receptor antagonist (IL-1ra).
- Alongside IL-6, exercise promotes the upregulation of IL-15 and brain-derived neurotrophic factor (BDNF), all of which help in regulating lipid and adipose tissue metabolism.
- Exercise reduces circulating levels of pro-inflammatory monocytes (i.e., CD14lowCD16+).
- Exercise has an indirect anti-inflammatory effect through the reduction of visceral fat and by promoting the secretion of myokines involved in muscle growth and repair (i.e., IL-15, IL-6, myostatin, IL-7, IL-4, and the leukemia inhibitory factor (LIF)).

Summary Points

- Type 2 diabetes mellitus (T2DM) is a noncommunicable chronic disease in which the lipid and inflammatory profiles play a major role in its pathophysiology and progression.
- One of the main hallmarks of T2DM is the accumulation of lipids in the liver and muscle when the buffering capacity of adipose tissue to store fatty acids is exceeded due to increased energy intake and reduced physical activity.

- Local adipose tissue dysfunction leads to impaired lipid profiles, characterized by elevated circulating levels of free fatty acids, as well as the accumulation of deleterious lipid intermediates including the diacylglycerols (DAG), sphingolipids (e.g., ceramides), and polar lipids (e.g., long-chain acyl-CoAs (LCA-CoA) and acylcarnitine).
- Local hypoxia and adipocyte apoptosis occurring in dysfunctional adipose tissue as well as the presence of lipid intermediates signal for the recruitment of immune cells, which are responsible for inducing the low-grade chronic inflammation associated with obesity-linked insulin resistance and T2DM.
- Exercise can counter lipotoxicity and improve overall insulin resistance through its action on the classic lipid biomarkers, intramuscular lipid characteristics, as well as on the lipid intermediates.
- Exercise can have both direct and indirect effects on the inflammatory milieu through reducing visceral adipose tissue by promoting a negative energy balance and through enhancing the secretion of anti-inflammatory myokines.
- The ultimate type, duration, frequency, and intensity of exercise for achieving the ultimate benefits on the lipid and inflammatory profiles in individuals with T2DM still remains to be elucidated.

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Part II

Circulating and Body Fluid Biomarkers: Impact of Diabetes on Pathology

Resistin as a Biomarker and Applications to Prediabetes

3

Seyfettin Üstünsoy

Contents

Introduction 69
Diabetes 69
Prediabetes: Definition and Related Diseases 70
The Role of Adipokines at Energy Balance 71
Resistin As a Biomarker at Different Types of Disease 72
The Association Between Resistin and Prediabetes and Outcomes 76
Conclusion 77
Key Facts of Resistin 78
Summary Points 78
References 78

Abstract

Resistin is a cysteine-rich protein, secreted from mature human adipocytes. Resistin has 108 amino acids. The resistin coding gene area is located on chromosome 19. It has regulatory role in many chronic inflammatory diseases, metabolic diseases, infectious diseases, and cancers. Also, it has modulatory effects on immunity systems via increasing or decreasing the secretion of cytokines, inflammatory and proinflammatory molecules from immune cells. In addition, it has also effects on cancer pathophysiology. To date, the exact role of resistin on metabolism is still limitedly known. The aim of this chapter is to summarize current knowledge about the prediabetes, clinical association of

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resistin on diseases, and disease-related mechanisms of resistin. Finally, this review highlights potential diagnostic and therapeutic roles of resistin in inflammatory diseases.

Keywords

Resistin · Prediabetes · Biomarker · Cytokine · Adipokine

Abbreviations

AD	Alzheimer's disease
ADA	American Diabetes Association
C	Carboxyl
CAC	Coronary Artery Calcification
CAP1	Cyclase-associated protein 1
CDC	Centers for Disease Control and Prevention
CRCs	Colorectal cancers
CRP	C-reactive protein
CVD	Cardiovascular disease
CVDs	Cardiovascular diseases
EMT	Epithelial to mesenchymal transition
FPG	Fasting Plasma Glucose
HbA1C	Glycosylated hemoglobin
HDL	High density lipoprotein
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL	Interleukin
IL-6	Interleukin-6
LpPLA2	Lipoprotein-associated phospholipase A2
Mg/dl	Milligrams per deciliter
MS	Metabolic syndrome
N	Amino
PPARγ	Peroxisome proliferator-activated receptor gamma
RELMs	Resistin-like molecules
SMM	Skeletal muscle mass
sol ICAM-1	Soluble intercellular adhesion molecule-1
sol TNF-R2	Soluble tumor necrosis factor (TNF) receptor 2
TC	Total cholesterol
TGs	Triglycerides
Type 1 DM	Type 1 Diabetes Mellitus
Type 2 DM	Type 2 Diabetes Mellitus
TZDs	Thiazolidinediones
USA	United States of America
VAT	Visceral adipose tissue
WHO	World Health Organization

Introduction

Resistin is a cysteine-rich protein, expressed in adipocytes. It has regulatory role in many chronic inflammatory diseases, metabolic diseases, infectious diseases, and cancers. Also, it has modulatory effects on immunity systems via increasing or decreasing the secretion of cytokines, inflammatory and proinflammatory molecules from immune cells. In addition, it has also effects on cancer pathophysiology. To date, the exact role of resistin on metabolism is still limitedly known. The aim of this chapter is to summarize current knowledge about prediabetes, clinical association of resistin on diseases, and disease-related mechanisms of resistin. Finally, this review highlights potential diagnostic and therapeutic roles of resistin in inflammatory diseases.

Diabetes

Diabetes is a lifelong chronic disease, which is one of the biggest pandemic public health problems around the world that ranks fifth among the causes of total deaths in the world (Pandey et al. 2015). It is defined as high blood sugar due to inadequate insulin secretion from pancreas beta cells or inadequate cellular response to the plasma insulin. Clinical diagnosis of diabetes is performed via American Diabetes Association (ADA) Criteria for Diabetes, and there are four types of diabetes categorized according to the etiological factors (ADA 2021). Underlying etiology mostly depends on high energy intake and low energy consumption–related situations like obesity and sedentary lifestyle; in addition, genetical and environmental factors also important at diseases development and progression (Moini 2019). Diabetes has a variety of complications, mainly defined in two groups as microvascular and macrovascular with regard to affected vessel diameter. Main microvascular complications are retinopathy, nephropathy, and neuropathy. The major macrovascular complications are strokes, coronary events, and amputations. Both of them are leading causes of morbidity and mortality of diabetic patients decreasing their life quality along with financial burden (Forbes and Cooper 2013). According to the WHO 2021 statistics, estimated diabetic people number increased to the 422 million around the world, generally living at middle or low income countries. Most of them are type 2 diabetics (WHO 2020). The last national diabetes statistics report published by Centers for Disease Control and Prevention (CDC) of United States of America (USA) clearly demonstrated the predicted economic burden of diabetes on USA economy. Health care spending of diabetes in USA rose by 26% between 2012 and 2017. Estimated total cost is reached nearly $403,9 billion annually (O'Connell and Manson 2019; CDC 2020). As a result, diabetes has devastating economic and health problems on both diabetic people with his/her family and public health, in addition governments.

Prediabetes: Definition and Related Diseases

We can simply define the prediabetic patients as whose blood sugar levels are neither at the normal level nor at the diabetic level according to the ADA/WHO criteria for diabetes. According to the ADA, diagnosis of prediabetes is based on the presence of impaired fasting glucose (IFG) (calculated fasting plasma glucose level is between 100 mg/dl and 125 mg/dl) or impaired glucose tolerance (IGT) (calculated second hour plasma glucose level is between 140 mg/dl and 199 mg/dl) during a 75-gram oral glucose tolerance test or hemoglobin A1c level between 5.7% and 6.4% (ADA 2014) (Table 1). The increasing prevalence of prediabetes is a major public health problem that is drawing attention all over the world. However, there is still a debate between WHO and ADA for the exact definition of this term. WHO advices intermediate hyperglycemia term instead of prediabetes, but ADA suggests prediabetes term along with declining top reference level of normal FPG level to the 100 mg/dl (WHO and International Diabetes Federation 2006; ADA 2014).

Detecting prediabetic people is very important, because like diabetic patients, there are close relationships between prediabetes and major public health diseases. In a previous ADA expert panel, the prediabetic individuals' estimated rate for progression to the diabetes was reported as 70% (Eikenberg and Davy 2013). Lightart et al. published a prospective chort study, supporting the ADA expert panel report that calculated the risk of prediabetic people at age 45 years developing diabetes as 74% (Ligthart et al. 2016). In addition, people at the prediabetic stage have high risk of developing cardiovascular pathologies when compared to the normoglycemic population. Myocardial infarction, congestive heart failure, and other coronary problems along with hypertension, obesity, and dyslipidemia prevelance is nearly threefold high in prediabetic population than normoglycemic population (Brannick and Dagogo-Jack 2018). Also, another leading cause of total deaths around the world is cancer, which is closely related with the prediabetes. Huang et al. published a recent meta-analysis of 16 prospective cohort studies including 891,426 participants. Their study revealed that prediabetes is a sole factor that increases the risk of cancer development. According to their study prediabetes was significantly associated with increased risks of cancer of the gastrointestinal system (stomach, colorectum, liver, pancreas), breast, and endometrium; nevertheless they could not find a statistically significant relationship with lung, prostate, ovary, kidney, and bladder cancer (Huang et al. 2014). Another important disease group related to the prediabetic is neurological disorders. The exact pathophysiology between

Table 1 Diagnostic criteria for prediabetes

Result	Fasting Plasma Glucose (FPG)	Oral Glucose Tolerance Test (OGTT)	HbA1C (HemoglobinA1c)
Normal	<100 mg/dl	<140 mg/dl	<5.7%
Prediabetes	100 mg/dl to 125 mg/dl	140 mg/dl to 199 mg/dl	5.7–6.4%
Diabetes	126 mg/dl or higher	200 mg/dl or higher	6.5% or higher

[a]Adopted from ADA report (2014)

hyperglycemia and major neurological disorders has not yet been entirely clarified; there are so many papers in literature that have shown the linkage between them indisputably. Especially dementia, Alzheimer's disease (AD), and depression are linked to hyperglycemic conditions. A recent study conducted on diet-induced prediabetic Sprague Dawley rats reported that HPA axis activity and response to stress was diminished, which increases the risk of depression development (Mosili et al. 2020; Willmann et al. 2020; Mihai et al. 2021). AD is a progressive brain disorder, occurring with advancing age. Main symptoms are loss of memory and cognition. Underlying mechanism is not obvious yet, but neuronal death due to the accumulation of toxic amyloid plaques and tau tangles could be the reason. Which factors are affecting the neurons and triggering the formation of these plaques is not known, however high blood glucose–related increased oxidative stress and corrupted blood–brain barrier are on the front line (Ahn and Song 2019; Marseglia et al. 2019). A recent study demonstrated how AD, type 1 DM, type 2 DM, and prediabetes are interlinked. Both AD and hyperglycemia amplified the brain tissue cytokine levels, which increase the neuronal injury, amyloid plaques, and tau tangles, and disturb the blood–brain barrier (Sankar et al. 2020).

Catching these patients at prediabetic level is crucial. Just like diabetes, prediabetes too is a huge public health pandemic around the world. However, we are lucky because if we can identify prediabetic people in the population with correct method as soon as possible, we can prevent most of the choric diseases like diabetes, AD, cardiovascular diseases, and we can decrease the health expenditures of countries along with deaths related to these pathologies.

Recent studies conducted on prediabetic patients revealed that just lifestyle modifications without medication prevented a high percentage of prediabetic patients developing diabetes, cardiovascular, and/or neurological pathologies (Pan et al. 1997; Aakko et al. 2001; Knowler et al. 2002; Ramachandran et al. 2006). Researchers evaluated anti-diabetic drug therapies with different activity mechanisms in population-based randomized, controlled trials and found out that the risk of developing diabetes and related pathologies decreased between 25% and 75% depending on the drug used (Knowler et al. 2002, 2005; Ramachandran et al. 2006; Gerstein et al. 2006; Vadini et al. 2020).

The Role of Adipokines at Energy Balance

Adipose tissue is a special kind of loose connective tissue that consists of mostly adipocytes. Also preadipocytes, fibroblasts, vascular endothelial cells, and adipose tissue macrophages are part of this tissue. The main role of adipose was known to be storing energy as fat, body heat isolation, and behave like a shock absorber against traumas until recent years (Trayhurn and Beattie 2021). After the discovery of leptin and adiponectin the adipose tissue is defined as an edocrine organ, because scientist understood that these bioactive molecules has very important roles on controlling human methabolism (Funcke and Scherer 2019). There are so many active molecules secreted from different parts of adipose tissue regulating a variety of important

metabolic/immunologic cascades (Halberg et al. 2008). The term "adipokine" is commonly used to describe the adipose tissue-derived proteins that have autocrine, paracrine, and endocrine affects (Li et al. 2017). Energy metabolism of human body is controlled through adipose tissue and skeletal muscle cells via adipokines and other secreted modulatory molecules. Excess energy yielded by diet is converted to the triglycerids stored in the white adipocytes or glycogen stored in the smooth muscle cells and liver. If this excess energy intake situation goes on, the person starts to gain weight. As a result, it turns into obesity. Obesity is a major risk factor for the development of insulin resistance and type 2 diabetes (Al-Goblan et al. 2014; Rodríguez et al. 2015). In obese individuals, adipokines, proinflammatory cytokines, increased oxidative stress, and related end products that are involved in the development of insulin resistance is increased. As a consequence of ruined lipid and carbohydrates metabolism, insulin resistance and type 2 diabetes occur (Burhans et al. 2018; Kusminski et al. 2016). Adipokines and myokines are the key regulatory molecules balancing and controlling this fragile metabolic equilibrium of lipid and carbohydrates metabolism. The main myokines secreted by skeletal muscle tissue are myostatin, interleukin (IL)-8, IL-15, irisin, fibroblast growth factor 21, and myonectin; adipokines secreted by adipose tissue are leptin, adiponectin, resistin, chemerin, and visfatin (Kita et al. 2019).

Resistin As a Biomarker at Different Types of Disease

The term biomarker refers to a biological molecule found at detectable levels in blood, other body fluids, or tissues that guides the health professionals to define the metabolic situation as normal or abnormal. Also biomarkers are commonly used for the follow-up of the disease condition or efficacy of the given treatment. The answer to "Why do we need biomarkers?" is that human metabolism is affected by many intrinsic or extrinsic factors causing variability from man to man, which is forcing decision making difficult about the clinical situation or efficacy of the given treatment. Therefore, we need to eliminate this conflicting situation where biomarkers take part in (Strimbu and Tavel 2010).

Resistin is one of the recently discovered adipokines. It was first described in 2001 in mice by Steppan et al. during examining the activity mechanism of thiazolidinediones (TZDs) (Steppan et al. 2001a). The resistin coding gene area is located on chromosome 19. It is a 12.5 kDa cysteine rich protein mainly secreted from adipocyte consisting of 108 amino acids. Besides adipocyte, monocytes, macrophages, and epithelial cells also produce resistin. It is a member of resistin-like molecules (RELMs) proteins family (Steppan et al. 2001b). These polypeptides consist of an amino (N) terminal sequence, a variable middle section, and a carboxyl (C) terminal. Their X-ray crystal structure resembles a jelly roll (Li et al. 2021). It is called as "resistin" because its expression has positive correlation to insulin resistance and obesity.

Adipose tissue is increasingly gaining the attention of researchers in the last years because of its unique properties and newly defined functions controlled by fat tissue secreted adipokines, proinflammatory, inflammatory cytokines, and other

active metabolites. There are many ongoing researches particularly about resistin's metabolic activities and its role in disease pathophysiology.

Resistin As a Biomarker in Cardiovascular Disorders

Obesity, diabetes, increased oxidative stress, and atherosclerosis are the leading causes of cardiovascular diseases. The common feature of these pathological situations is slowly progressing low-grade inflammatory state of the body that causes endothelial damage in the heart, brain, or other body parts' arteries where plaque formation is triggered, and that results in atherosclerosis. The exact cause of atherosclerosis is still not known. However, there are many previous studies in the literature revealing the impact of this inflammatory state on the atherosclerotic cardiovascular diseases (CVDs) (Insull 2009).

A recent study suggests that serum resistin levels are positively correlated with serum inflammation markers interleukin-6 (IL-6), soluble tumor necrosis factor (TNF) receptor 2 (sol TNF-R2), and soluble intercellular adhesion molecule-1 (sol ICAM-1), lipoprotein-associated phospholipase A2 (LpPLA2), C-reactive protein (CRP), and coronary atherosclerotic plaques. The study was conducted on 879 asymptomatic, nondiabetic subjects whose resistin levels were compared to 215 type 2 diabetic subjects and healthy control group. Serum resistin levels were increased in both experiment groups compared to the control group. Additionally, Coronary Artery Calcification (CAC) is assumed as the evidence of coronary atherosclerosis. Postmortem experiments' results also supported this evidence. In this study plasma resistin levels were found significantly associated with CAC in the first group subjects (Reilly et al. 2005).

Sabry et al. designed an animal experiment in male albino rats. Experiment groups categorized as obese, atherosclerotic, and control groups and divided two subgroups, each primary group as peroxisome proliferator-activated receptor gamma (PPARγ) agonist administered and not administered. The study demonstrated a significant increase in plasma resistin levels accompanied with raised proinflammatory and inflammatory markers in both untreated obese and atherosclerotic subgroups. Their results revealed a significant positive correlation between resistin levels and serum levels of total cholesterol (TC), triglycerides (TGs), and a significant negative correlation between its levels and serum levels of high density lipoprotein (HDL). Histopathological analysis demonstrated the severe atherosclerotic plaques in obese and atherosclerotic group with both treated and untreated subgroups compared to the control group. PPARγ administration slightly reversed the atherosclerosis progression (Sabry et al. 2020).

There are many immunomodulators interfering the atherosclerotic cascade. As mentioned above resistin is a key regulator of insulin resistance and inflammatory processes. For this reason, it is thought that resistin could be a good biomarker candidate molecule either for diagnosing the CVDs or evaluating the success of the treatments. Moreover, these findings suggest that resistin could be a good target for designing drugs against CVDs.

Resistin As a Biomarker in Obesity

Obesity is a global serious health problem related to the increased energy intake and fat deposition, Causing physical and psychological problems accompanied with

significant decline in lifetime expectancy and quality of life. It is a chronic energy metabolism disorder associated with environmental, psychological, and genetic factors. Obesity is strongly linked to the oxidative stress–related inflammation and insulin resistance, which are causally related to the several chronic metabolic disorders (Thaler and Schwartz 2010).

Different reports have described the effect of resistin on obesity and related metabolic disorders. Medina et al. performed an observational study with undergraduate students and adult workers. They evaluated the relationship between resistin and anthropometric measurements, body composition, and biochemical tests. According to their study results, there is a correlation between resistin and increased visceral adipose tissue (VAT), skeletal muscle mass (SMM) and metabolic syndrome (MS) (Nájera Medina et al. 2019). Moreover, in another study, elevated resistin levels were observed significantly higher in abdominal obese patients than in patients without abdominal obesity or in the control group (Montazerifar et al. 2016). Consistent with the previous studies, a recent clinical trial showed the circulating level of resistin was increased in hypertensive subjects with obesity compared to those in subjects with normal body weight (Kravchun et al. 2020). Association of resistin with inflammatory indicators may suggest that, in the light of above studies, proinflammatory role of resistin massively takes part in the development of obesity and related metabolic events. In conclusion, this novel biomarker may be important in preventing obesity and related morbidities and mortalities.

Resistin As a Biomarker in Diabetes

Diabetes is the most common endocrinological pathology among the adults in the industrialized countries up to 10% of general population. Most of them are type 2 diabetic people. Type 2 DM and related mortalities-morbidities are the leading cause of deaths around the world (Forbes and Cooper 2013; Knowler et al. 2002). Besides, type 2 DM and related mortalities-morbidities constitute a great economical burden. For diagnosing these people earlier before the complications occurred or preventing type 2 DM development we need new biomarkers. Resistin is an optimum candidate for this purpose because of its unique properties resembling the insulin and particular metabolic activities of adipose tissue and skeletal muscle mass, which are major actors of fat and glucose turnover processes. Furthermore, resistin could modulate systemic inflammation and atherosclerosis in type 2 DM through stimulation of the proinflammatory cytokines secretion from immune system cells.

Previous studies explored that resistin levels are higher in type 2 diabetic people compared to the healthy individuals, positively correlated with BMI of the type 2 diabetics while particularly depending on the increased inflammatory cytokine release (Jung and Choi 2014; Su and Peng 2020). A recent retrospective clinical trial conducted in Italy have put forward that about 50% of healthy obese people will develop dysglycemia condition and about 20% will develop type 2 diabetes mellitus within 8 years in their future. In addition, serum resistin levels were found to be high in type 2 DM group compared to the euglycemic and dysglycemic patients during the 8 years follow-up. According to the results of this study, authors suggested that higher levels of resistin is a warning sign in obese people for developing type 2 DM,

even years before the disease onset (Derosa et al. 2020). Moreover, Kapłon-Cieślicka et al. investigated the relationship between the resistin levels and type 2 DM progression. Their study outcome unveiled that high resistin level is a bad prognostic parameter in this patient group and resistin concentration more than 11 ng/mL might be a predictive indicator of increased risk of death in long-term follow-up (Kapłon-Cieślicka et al. 2019).

Resistin As a Biomarker in Cancer

Cancer is a major public health problem worldwide and the second leading cause of death in the world. It is defined simply as the abnormal cell growth at different parts of the body. Depending on the cancer cell type it could be defined as invasive or noninvasive. Signs and symptoms and onset of time and place show great variety according to the cancer type and location. Prostate, lung, and bronchus and colorectal cancers (CRCs) are the most common cancer types in men; however majority of the cases are prostate cancer accounting for 26% of all cancers. For women, breast cancer, lung, and CRCs are the most common cancer types in women. Breast cancer is the most frequently detected type as accounting for 30% all new diagnoses (Siegel et al. 2021).

Cancer development is a multifactorial event. Although, it is not easy to find out the exact reason why some people have cancer some not, there are many risk parameters defined by scientist via epidemiological studies. Some of them are avoidable and some of them are not. It is possible to decrease the cancer risk of a person by restricting the exposure to that risk factor. Major well-defined risk factors are age, family history and genetic factors, alcohol and tobacco consumption, chemicals, chronic Inflammation, diet, obesity, radiation, sunlight, and etc.

Chronic inflammation accompanied with unhealthy diet-linked pathologies like obesity, type 2 DM, increased oxidative stress, etc., provoke the cellular dysplasia that may result in cancer development due to complex intra- and extracellular signaling molecules. Because of this close relationship of cancer and low-grade chronic inflammatory state, it is thought that resistin could be an important modulatory molecule in cancer cascade. As a result it can be used as a biomarker for the cancer identification, follow-up the treatments, or risk stratifications (Sudan et al. 2020; Deb et al. 2021).

Breast cancer is the most prevalent cancer in women and the second leading cause of death in women after CVDs. Obesity is one of the major risk factors along with familial history in breast cancer, as mentioned before fat tissue has very important modulatory effects on cancer development and malignancy via the release of pro-inflammatory cytokines and adipocytokines. Besides that, breast tissue has huge amount of fat, which augments the effects of fat tissue secreted molecules in breast cancer. Previous studies showed the adverse effects of obesity clearly. Obese breast cancer patients are more prone to disease recurrence and metastasis. Their prognosis is bad and chemotherapy and hormone therapy is less effective, and they have more surgical complications (Brown 2021). Lastly, Assiri et al. published a study evaluating the diagnostic and predictive value of serum adipokines breast cancer. They have enrolled in their study 298 postmenopausal females consisting of three groups:

breast cancer patients, healthy control, and females with benign breast lesion. According to their results, serum resistin levels were increased significantly compared to the healthy control and females with benign breast lesion (Assiri and Kamel 2016). Consistent with the previous study, HOU et al. found serum resistin levels significantly increased in patients with breast cancer compared to the healthy controls, and also found that serum resistin levels were correlated with the size of tumor size (Hou et al. 2007; Zeng et al. 2020). Moreover, resistin provokes the metastatic potential of breast cancer cells via increasing epithelial to mesenchymal transition (EMT) and stemness through cyclase-associated protein 1 (CAP1) (Avtanski et al. 2019).

Prostate cancer is the second most common cancer type in men. It is the cancer of prostate gland. It grows slowly. Aging, family history, and obesity are the major risk factors. Mortality rate of prostate cancer in obese men is calculated 34% higher according to the normal weight men (Calle et al. 2003). In vivo studies demonstrated the direct effect of resistin on prostate cancer development and progression. It stimulates the PI3K/Akt signaling pathway-related cascades in prostate cancer cell proliferation (Kim et al. 2011). On the other hand, Ardalan et al. could not find a correlative data in their research and hypothesized that serum adipokines could be used to choose the correct patients together with the clinical variables and reduce the unnecessary biopsy rates in patients (Ahmad et al. 2019).

In addition to the breast and prostate cancer, there are many researches dealing with the adipokines and their effects on different cancer types' development and progression, and related cellular signaling pathways. To find out the direct linkage between them is extremely important. Because, by this way we could use the serum adipokine levels as cancer biomarkers before the onset of the disease or we could invent new drug molecules that are blocking or activating the signaling pathways taking part in the cancer pathophysiology.

The Association Between Resistin and Prediabetes and Outcomes

Even though, studies over the past years have made significant contributions to the field of research related to resistin, the specific biological effects of resistin is not totally revealed yet. However, growing data in the literature absolutely implicated the modulatory and regulatory impacts of resistin in health and disease states. The main metabolic effects of resistin are proinflammatory and inflammatory immune response against low-grade progressive inflammations like atherosclerosis, insulin resistance, obesity, heart disease, etc. It has proliferative effects on cells (Kim et al. 2011). According to the ADA, diagnosis of prediabetes is based on the presence of impaired fasting glucose (IFG) or impaired glucose tolerance or hemoglobin A1c level between 5.7% and 6.4% (American Diabetes Association 2014).

Obesity and type 2 DM are important risk factors for colorectal cancer development. Epidemiological studies have shown that compared to the normal weight people type 2 DM have a higher risk of developing colorectal cancer. There are different contributing factors related to the metabolic syndrome, insulin resistance

and adipocyte secreted adipocytokines seem to be of great importance (Campbell et al. 2010). Likewise, prediabetic individuals are also at high risk of having colon cancer. It has been demonstrated by previous studies that adipose tissue-derived adipocytokines have direct effects on polyp development. Therefore they are thought to be an independent risk factor for polyps in prediabetes subjects (Cha et al. 2013).

Previous studies have showed that prediabetic people are more prone to the CVDs, and also higher resistin levels were measured among the CVD patients (Nimptsch et al. 2019). According to the OPERA study results, the highest serum resistin levels were calculated among prediabetics, especially among IGT subjects that implicate resistin as a marker for atherosclerosis in prediabetic people. This is a population-based, epidemiological study aiming to find out the risk factors and disease endpoints of atherosclerotic CVDs (Galla et al. 2020). A recent study conducted on 88 voluntary participants revealed that resistin levels in prediabetic patients were significantly higher than the control group. In addition, resistin concentrations positively correlated with diabetic risk factors such as age, fasting blood glucose, HbA1c, HOMA-IR, OGGT 0, OGGT second hours. In the light of their study outcomes, they suggest that increased resistin levels could be a marker for the development of insulin resistance in prediabetic individuals (Bilgetekin et al. 2019).

Chronic inflammation accompanied with unhealthy diet or excessive energy intake–linked pathologies like obesity, type 2 DM, increased oxidative stress, cancer, etc., also occurs during prediabetic stage. Nevertheless, intervening at prediabetic phase via eliminating the risk factors or the causative reasons reverse the pathophysiological cascades; finally disease development or progression could be prevented. By that way, the quality of life of people would be better and economical burden of governments would be eased.

Conclusion

Resistin is a cysteine-rich inflammatory cytokine, which is widely researched in the recent years. It has unique properties and newly defined functions. Resistin is an important modulatory and regulatory peptide for several metabolic activities. Increasing data indicates that resistin plays important regulatory roles in diabetes, prediabetes, obesity, CVDs, and atherosclerosis. Adipose tissue secreted resistin stimulates the immune system cells and increases inflammatory and pro-inflammatory cytokine release that leads to inflammation, endothelial dysfunction, thrombosis, and low-grade chronic inflammation, which results in disease initiation and evolution. Recent date revealed the significance of resistin in cancer pathogenesis and therapeutic outcomes. In the light of growing data resistin could be a potential clinical biomarker in prediabetic people. It has close relationship to the above mentioned chronic disease and increased plasma levels calculated. Previous data in the literature exposed that intervening at prediabetic phase via eliminating the risk factors or the causative reasons reverse the pathophysiological cascades, and the development or progression of the disease could be prevented. Moreover, we could use the serum resistin levels as biomarkers of diseases or at-risk stratification by

defining the cutoff limits in the prediabetic stage. Understanding the role of resistin and cellular signaling pathways will contribute to the better opportunities for the diagnosis and treatment of pathologic states. On the other hand, we could invent new drug molecules, which are blocking or activating the signaling pathways taking part in the disease pathophysiology.

Key Facts of Resistin

Resistin is a cysteine-rich inflammatory cytokine, which is widely researched in the last years.

Resistin plays important regulatory roles in diabetes, prediabetes, obesity, CVDs, and atherosclerosis.

Serum could be a biomarker of diseases in the prediabetic stage.

Resistin levels of prediabetic patients are higher and detectable limits.

Summary Points

Resistin is an important modulatory and regulatory peptide at several metabolic activities.

Resistin stimulates immune system cells' cytokine release.

Cytokines lead to inflammation, endothelial dysfunction, thrombosis, and low-grade chronic inflammation.

That triggers disease initiation and evolution.

At prediabetic phase, via eliminating the risk factors, the development or progression of the disease could be prevented.

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4 Altered Metabolome of Amino Acids Species: A Source of Signature Early Biomarkers of T2DM

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Contents

Introduction 86
Diabetes Mellitus 87
The Need for New Biomarkers for Diabetes 88
Amino Acids in Metabolic Signaling and Insulin Resistance 89
Altered Amino Acid Profiles in Insulin Resistance and Diabetes 91

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Amino Acids Metabolic Intermediates As Markers for Insulin Resistance and Diabetes 118
Conclusions 119
Applications to Prognosis, Other Diseases, or Conditions 120
Mini Dictionary of Terms 120
Key Facts of Amino acids 121
Summary Points 121
References 122

Abstract

Diabetes mellitus is a chronic metabolic disease with serious health consequences for a modern civilization that often lead to premature death. With the rapid increase in the number of people diagnosed with type 2 diabetes, early identification of those individuals at higher risk of progression to diabetes is a key criterion enabling the timely intervention or treatment. In recent years, omics-based technologies have given us unprecedented insight into circulating biomarkers in common diseases. Branched-chain amino acids: valine, leucine, isoleucine, and aromatic amino acids, that is, tyrosine and phenylalanine, have been demonstrated as the most consistent metabolite biomarkers for diabetes, in particular type 2.

Therefore, amino acids quantification in biological material, primarily in plasma could be a valuable prognostic tool for determining metabolic abnormalities leading to this disease. Revealing these interactions and possible mechanisms may prove beneficial for the prediction and treatment.

Keywords

Amino acids · Branched-chain amino acids · Insulin resistance · Prediabetic state · Diabetes · Type 2 diabetes mellitus · Metabolic disease · Biomarker · Metabolomics

Abbreviations

2-AAA	2-aminoadipic acid
2-h PG	2-h plasma glucose test
3-HIB	3-hydroxyisobutyrate
AAAs	Aromatic amino acids
AAs	Amino acids
AILS	AminoIndex LifeStyle diseases test
AKT	Protein kinase B, PKB
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BAIBA	β-aminoisobutyric acid
BCAAs	Branched-chain amino acids
BCKA	Branched-chain α-ketoacid
BCKDC	Branched-chain α-ketoacid dehydrogenase complex

BCKDH	Branched-chain α-ketoacid dehydrogenase
BHBA	3-hydroxybutyrate
BMI	Body Mass Index
Cit	Citrulline
CoA	Coenzyme A
CVD	Cardiovascular disease
Cys	Cysteine
DAG	Diacylglycerol
DM	Diabetes mellitus
FAAs	Free amino acids
FFAs	Free fatty acids
FGF21	Fibroblast growth factor 21
FHS	Framingham Heart Study
FOXO	Forkhead Box O transcription factor
FPG	Fasting plasma glucose
GC	Gas chromatography
GDM	Gestational Diabetes mellitus
GDR	Glucose disposal rate
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GSK-3	Glycogen synthase kinase-3
HbA1c	Glycated hemoglobin
HECP	Hperinsulinemic-euglycemic clamp procedure
His	Histidine
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
IDF	International Diabetes Federation
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
Ile	Isoleucine
Ins120 min	2-h post-challenge insulin
IR	Insulin Resistance
IRAS	Insulin Resistance Atherosclerosis Study
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
LC	Liquid chromatography
Leu	Leucine
L-GPC	linoleoyl- glycerophosphocholine
LPC	Lysophosphatidylcholine
MDC	Malmö Diet and Cancer Study
MetS	Metabolic syndrome
METSIM	Metabolic Syndrome in Men Study
mmBCFA	Monomethyl branched-chain fatty acids
MS	Mass spectrometry
mTORC1	Mammalian target of rapamycin complex 1

NEFAs	Non-esterified fatty acids
NGT	Normal glucose tolerance
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
Orn	Ornithine
PCs	Phosphatidylcholines
PFAAs	Plasma-free amino acids
Phe	Phenylalanine
PPARα	Peroxisome proliferator-activated receptor α
Pro	Proline
QMDiab	Qatar Metabolomics Study on Diabetes
RISC	Relationship of Insulin Sensitivity to Cardiovascular Risk study
ROS	Reactive oxygen species
RQ	Resting respiratory quotient
SABRE	Southall And Brent REvisited Study
Ser	Serine
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TCA	Tricarboxylic acid cycle
TKR	Tyrosine kinase receptor
Trp	Tryptophan
TüF	Tübingen Family study for T2DM
Tyr	Tyrosine
UCD-T2D	University of California-Davis T2DM rat model
Val	Valine
VFA	Visceral fat area
α-HB	α-hydroxybutyrate
α-KB	α-ketobutyrate

Introduction

Amino acids are defined as organic compounds containing amino($–NH_2$) and carboxyl(–COOH) functional groups along with a side chain (R group) specific to each amino acid (AA). They are referred as the building blocks of proteins needed for animal nutrition and basic units for synthesis of hormones and neurotransmitters (Lopez and Mohiuddin 2021). Human proteins are made up of 20 AAs, 9 of which are considered “essential” because they cannot be synthesized from other metabolites in the human body (White and Newgard 2019). AAs play a key role in many metabolic pathways, and quantification of free amino acids (FAAs) in biological fluids and tissues has historically provided nutritional information used in the diagnosis of various diseases, particularly metabolic impairments (Nagao and Kimura 2020).

During the last decade, many studies have consistently reported the positive association of plasma or serum FAAs with insulin resistance (IR) and diabetes in

individuals from different ethnic groups and with varying degrees of obesity in large prospective and cross-sectional human studies (Newgard et al. 2009; (Giesbertz and Daniel 2016; Chen et al. 2019; Bi and Henry 2017). Most of these publications demonstrated that increased levels of branched-chain amino acids in plasma, serum, and also in urine (Branched-chain amino acids (BCAAs); valine, leucine, isoleucine) are associated with obesity, IR, and diabetes, in particular type 2 diabetes mellitus (T2DM) (Newgard et al. 2009; Shah et al. 2012; McCormack et al. 2013; (Würtz et al. 2013; Bi and Henry 2017; Nie et al., 2018; Yousri et al. 2015).

Hyperaminoacidemia observed in obesity may be related to increased IR. Insulin resistance is believed to reduce the BCAA catabolism by suppressing the enzymatic activity of branched-chain alpha-keto acid dehydrogenase complex (BCKDC), which is considered as the plausible mechanism explaining the increased BCAA levels in obese or diabetic individuals (Bi and Henry 2017). Elevated BCAA levels have often been shown to predict the development of T2DM well in advance of its actual occurrence, what is very interesting from a diagnostics standpoint (Wang et al. 2011; Yamakado et al. 2015; McCormack et al. 2013; Ianni et al. 2017).

In addition, there is a body of evidence suggesting the correlation between IR and changes in aromatic amino acids (AAAs), that is tyrosine and phenylalanine, as well several other AAs (Bi and Henry 2017; Chen et al. 2019). Therefore, AAs quantification in biological material, primarily in plasma, may be a useful indicator of the presence of metabolic abnormalities leading to diabetes. This chapter reviews molecular and clinical associations between altered AA levels and diabetes development and interprets underlying biochemical mechanisms.

Diabetes Mellitus

Diabetes mellitus (DM) is a highly prevalent chronic metabolic disease with major health implications for a modern civilization (Freitas et al. 2017; Gan et al. 2020), and the eighth major mainstay of mortalities around the globe (Hameed et al. 2020). It is characterized by an increase in blood glucose level and inability to utilize glucose in adipose and muscle tissues (Zhao et al. 2017; Al-Abbasi 2012). Among the three main types of DM, i.e.,: type 1 (T1DM); type 2 (T2DM); and gestational (GDM), the T2DM is most commonly diagnosed (about 90% of cases) ("Diagnosis and Classification of Diabetes Mellitus," 2004; Gan et al. 2020).

T2DM is characterized by abnormal glucose and lipid metabolism, resulting from resistance to the effects of insulin and insufficient response to the secretion of this hormone. As the disease progresses, there may also occur a partial β-cell insufficiency and deficiency in insulin production. ("Diagnosis and Classification of Diabetes Mellitus," 2004; Gan et al. 2020; Chen et al. 2019).

According to the International Diabetes Federation (IDF) data, the global diabetes incidence has continuously increased each year, with an estimated 578 million cases by 2030 and 700 million people diagnosed with diabetes by the year 2045. Moreover, in 2019 one in two (50.1%) people living with diabetes were undiagnosed (Saeedi et al. 2019).

Chronic hyperglycemia is a common feature related to all DM subtypes that may lead to long-term damages (Association 2016; Freitas et al. 2017) and several vascular, neurological, immunological, and biochemical pathological changes (Al-Abbasi 2012). As the disease progresses, it is accompanied by multiple complications and dysfunction of various organs (Association 2016). Most commonly occurring is microvascular complications, e.g., diabetic retinopathy, nephropathy, and neuropathy; macrovascular complications, including coronary atherosclerotic heart disease with increased risk of cardiovascular events (Rawshani et al., 2017), and vascular disease of the lower extremities (peripheral artery disease), which is a leading cause of nontraumatic limb amputations (Vamos et al. 2010), as well as diabetic nephropathy, hypertension, and cerebrovascular disease, (Zhao et al. 2017). It is also associated with hypercholesterolemia, obesity, and other nutritional disorders (Al-Abbasi 2012).

Diabetes, particularly type 2, imposes a heavy financial strain on health care systems everywhere and shortens the life expectancy of diabetic patients (*International Diabetes Federation (IDF) (*2015*) Diabetes Atlas. 7th Edition, International Diabetes Federation, Brussels, Belgium. – References – Scientific Research Publishing*, no date), (Association 2016; Freitas et al. 2017; Zhao et al. 2017). The global T2DM prevalence is rising rapidly, particularly among those living in low- and middle-income countries (Ahola-Olli et al. 2019; Gan et al. 2020). T2DM is associated with increased mortality risk and reduced health-related quality of life, causing an immense social costs burden (Ahola-Olli et al. 2019; Chen and Gerszten 2020). The still high global incidence of T2DM and the accompanying increase in the number of its complications require earlier diagnosis and more effective treatment of this disease (Zhao et al. 2017).

The Need for New Biomarkers for Diabetes

Metabolic disorders are often present for years before becoming clinically apparent. In the early stages of T2DM, patients may have difficulties to recognize any symptoms of the disease (Zhao et al. 2017). By the time that relative insulin deficiency manifests as hyperglycemia and a T2DM is diagnosed, significant pancreatic β-cell failure already occurs. Early identification of individuals at higher risk of progression to diabetes allows the timely intervention to delay or prevent diabetes onset (Wang et al. 2011; Ahola-Olli et al. 2019).

Currently, most early screening and diagnostic methods for DM are directly related to glucose levels (Gan et al. 2020), which include fasting plasma glucose (FPG) measurement or 2-h plasma glucose (2-h PG) measurement in 75-g oral glucose tolerance (OGT) test (Association 2018; Gan et al. 2020). Moreover, the glycated hemoglobin (HbA1c) test is also often used to diagnose diabetes, providing an overall picture of average blood glucose levels over a period of past 3 months. In addition, several other clinical and laboratory predictors could be used in gauging diabetic status, such as body mass index and c-peptide measurement (Wang et al. 2011; Al-Abbasi 2012).

Meanwhile, many overweight to moderately obese people are found to have completely normal fasting plasma glucose and hemoglobin A1c levels, making them undiagnosed as prediabetic despite underlying metabolic abnormalities (Bi and Henry 2017). Therefore, there is a great need for biomarkers allowing an early diagnosis of prediabetic or diabetic patients (Wang et al. 2011).

In recent years, omics-based technologies have given us unprecedented insight into circulating biomarkers of common diseases. Application of metabolomics allowed identification of biochemical changes occurring prior to the onset of diabetes and provided additional information about pathophysiological mechanisms leading to DM. Amino acids and other metabolites were proposed as predictive markers indicating early metabolic perturbances. Diagnosis of patients at risk of diabetes onset is crucial to introduce changes in life style and prevention of disease development (Würtz et al. 2013; Guasch-Ferré et al. 2016; Ahola-Olli et al. 2019; Wang et al. 2011).

Amino Acids in Metabolic Signaling and Insulin Resistance

Despite the relevant role of the glucose-related pathways, here we are concentrating mostly on the protein metabolism influence on IR development. Many amino acids, especially BCAAs, are important nutritional signals that possess direct and indirect effects (Lynch and Adams 2014). The BCAA metabolic pathway crosses with the mechanism for IR (George 2017).

Evidence that BCAAs may not only have a "reporter quality" but may also contribute to IR and T2DM comes from cell culture and animal studies that propose sustained activation of complex 1 (mTORC1) (Newgard et al. 2009; Giesbertz and Daniel 2016). Proposed mechanisms (explaining how increased levels of BCAAs might be linked to metabolic disease) involve stimulation of the mTOR/p70S6K pathway and phosphorylation of IRS-1 at multiple serine sites (Nie et al. 2018). In this context, leucine is known to activate the nutrient sensing complex, mTORC1, which results in uncoupling of insulin signaling at an early stage of IR and other metabolic disorders. However, numerous observations indicate that BCAA-mediated mTORC1 activation is not necessary or sufficient to induce IR, and subsequent metabolic dysfunction, (Lynch and Adams 2014; Yoon 2016). Recently, in a rat model of diabetes (University of California-Davis T2DM rat model, UCD-T2D) an untargeted metabolomics study was performed showing that elevated plasma BCAA levels were not observed until 6 months after the onset of diabetes. This rules out the causal role of BCAAs in the occurrence of T2DM in the studied model (Yoon 2016; Biswas et al. 2019).

Zhao et al. (2020) worked with high-fat diet-induced obese (DIO) mice. Supplementation of these animals with BCAAs leads to heavy hepatic metabolic disorders, such as suppressed lipogenesis and increased glucose production. They also found that this impairs hepatic AKT2 signaling. BCAA supplementation stops AKT2 activation through mTorc1- and mTorc2-dependent pathways and promotes AKT2 degradation. As a matter of fact, the signaling pathways are other key elements in the development of IR. AKT is responsible for the transcription factors Forkhead box O (FOXO) activation, regulating the energy metabolism. FOXOs modulate the adipogenesis

process in the adipose tissue, they retain the beta cells function during oxidative stress, avoiding their replication, and they are a candidate as regulators of glucose production in the liver (Gross et al. 2009). Tyrosine kinase receptor (TKR) is an insulin receptor (IR), whose first targets are Insulin Receptor Substrate (IRS) 1, 2, 3, and 4. To better understand the most important targets, scientists knocked down these receptors in mice. While the knockdown of IRS3 or 4 showed no or limited effects on mice, knocked down IRS1 or 2 developed, respectively, IR and T2DM (Kubota et al. 2017).

An alternative mechanism referred to the BCAAs dysmetabolism suggests that deficiencies in BCAA metabolism are linked to IR and T2DM by the accumulation of BCAA levels in plasma, as well possibly toxic intermediates (Yoon 2016). BCAAs inefficient metabolism or incomplete oxidation, especially isoleucine and valine can result in anaplerotic stress and an imbalance between anaplerosis and cataplerosis that might cause suboptimal mitochondrial function in states of T2DM. For example, reduced mitochondrial branched-chain α-ketoacid dehydrogenase (BCKDH) activity results in the accumulation of branched-chain α-ketoacid (BCKA) and α-ketobutyrate (α-KB), which result in the restriction of propionyl-Coenzyme A (CoA)-derived metabolites to tricarboxylic acid (TCA) cycles, inducing anaplerotic stress and decreased amino acid fuel delivery to mitochondria (Fiehn et al. 2010; Adams et al. 2009; Yoon 2016).

Incomplete mitochondrial oxidation is thought to be the major cause for increased BCAA concentrations, independently of established IR. In obese subjects, the adipose tissue is characterized by an accumulation of fat, an increased pro-inflammatory state, and alterations in hormone and cytokine secretion that may strongly affect the mitochondrial function in peripheral tissues (Ianni et al. 2017).

In the adipose tissue of obese and T2DM patients with IR, as well in models of obesity in rodents, the expression of genes encoding BCAAs-metabolizing enzymes is significantly downregulated by an undefined mechanism compared to metabolically healthy controls – leading to elevated plasma BCAA levels (Lackey et al. 2013; Lynch and Adams 2014; Yoon 2016). Indeed, defective BCAA oxidation results in consequent accumulation of branched-chain keto acids and branched-chain fatty acids in peripheral tissues (Ianni et al. 2017). A high level of free fatty acids (FFAs) drives tissues, such as liver and skeletal muscles, toward IR, promoting the accumulation of such bioactive lipids as diacylglycerol (DAG) and ceramide (Zabielski et al. 2019). DAG accumulation impairs insulin signaling in liver (Fig. 1). Impaired insulin signaling in muscle results in increased proteolysis, which contributes to the release of BCAAs into the circulation and further supply of substrates for mitochondrial oxidation (Ianni et al. 2017).

However, the metabolism of BCAAs throughout the body is highly dependent on other organs; the expression of these enzymes in other organs such as the liver and muscle must be considered. Growing experimental evidence has posited that these impairments in the ability to metabolize BCAAs in adipose tissue may extend to other tissues (Lynch and Adams 2014). Expression of genes encoding enzymes of BCAA metabolism was downregulated in muscle and liver tissue of T2DM patients. Similar results were obtained in rats (Shin et al. 2014; Yoon 2016).

Zhou et al. (2019), using an integrative pathway analysis in human and mouse populations, showed that IR induced by obesity is connected with the modulations of

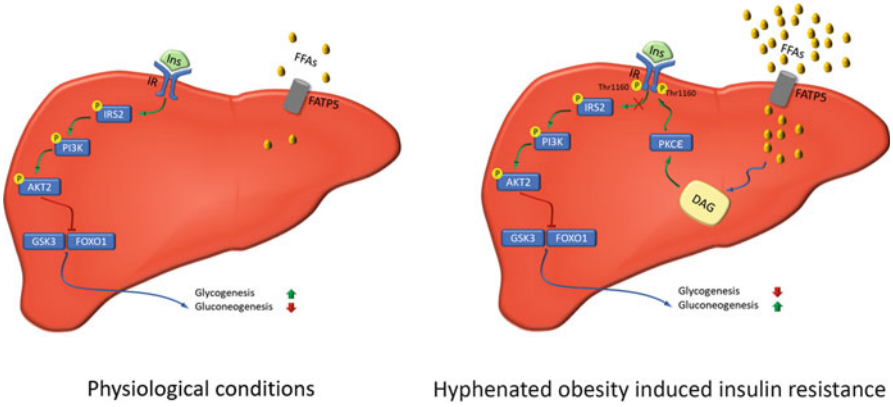

Fig. 1 Diacylglycerol (DAG) accumulation impairs insulin signaling in the liver. In physiological conditions, insulin arrives at the liver, activating the insulin receptor (IR) and starting a signaling cascade. This leads to the sequential activation of insulin receptor substrate 2 (IRS2), phosphatidylinositol-3-kinase (PI3K), and protein kinase B beta (AKT2). AKT2, inhibiting glycogen synthase kinase 3 (GSK-3), promotes the activation of glycogen synthase, increasing glycogenesis. AKT2 inhibition of Forkhead box protein O1 (FOXO1) suppresses the transcription of glucose 6-phosphatase, lowering gluconeogenesis. In the presence of a nonregulated amount of NEFAs in the plasma, they enter the liver through the fatty acids transport protein 5. This brings to an accumulation of DAG in the liver. DAG promotes the membrane translocation of PKCε, an isoform of PKC responsible for the phosphorylation of the Thr1160 on the insulin receptor. The conformational changes in the kinase active site impair its signaling function, leading to a loss of regulation on glycogenesis and gluconeogenesis

genes of the BCAA catabolism. They demonstrated that the BCAA catabolism is impaired in the obese state. In obese mice, restoring the catabolism of BCAAs lowers their levels and improves insulin sensitivity.

On the other hand, this defective enzymatic activity in adipose tissue could be compensated for by increased BCKDC activity in the liver (Lynch and Adams 2014; Yoon 2016). As a result, some individuals may be characterized by a more global reduction in the capacity to metabolize BCAAs, which may contribute to an increase in circulating BCAA concentrations to higher ranges that is related to the development of future T2DM and IR (Shin et al. 2014; Lynch and Adams 2014).

Contribution of BCAAs to IR is a complex process in which multiple, not fully understood, mechanisms are involved. A schematic presentation of possible mechanisms by which BCAAs contribute to development of IR is depicted on Fig. 2.

Altered Amino Acid Profiles in Insulin Resistance and Diabetes

In 1969, Felig et al. (1969) found that of 20 plasma amino acids measured, the concentrations of the three BCAAs: valine, leucine, isoleucine, and aromatic amino acids, phenylalanine and tyrosine, were increased, and glycine was lowered in plasma of obese subjects compared with age- and sex-matched lean individuals.

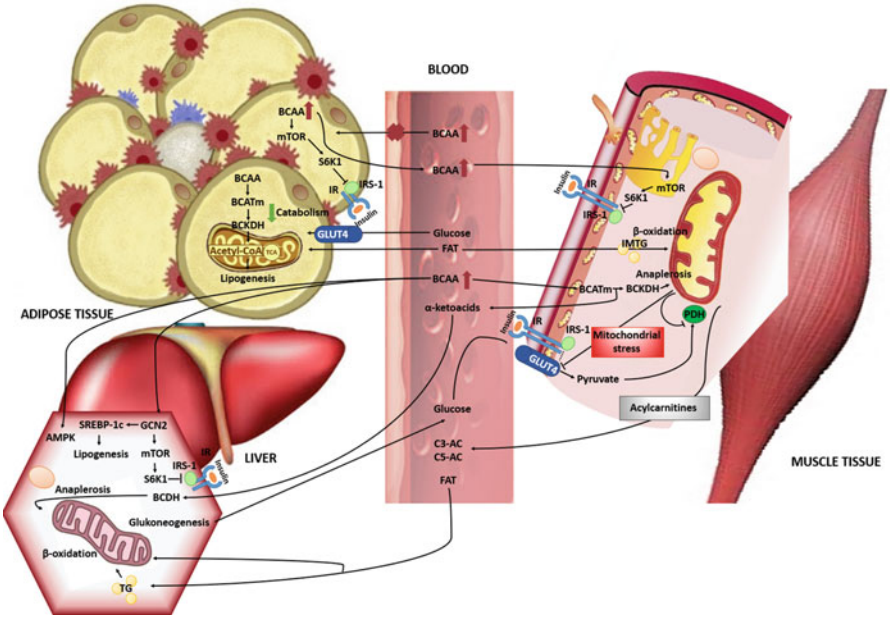

Fig. 2 A schematic view of possible mechanisms by which BCAAs contribute to development of insulin resistance. The first of proposed mechanisms involves stimulation of the mTOR/p70S6K pathway. High BCAA levels therefore inhibit IRS-1 and may impair insulin signaling. This causes cells to become less responsive to the secretion of insulin, resulting in insulin resistance

The concentration of each of the amino acids elevated in obesity correlated directly with serum insulin, suggesting that this increase was a manifestation of IR. In 2009, Newgard et al. (2009) replicated these findings of increased circulating BCAAs, tyrosine, and phenylalanine, and decreased glycine in obese insulin-resistant subjects compared to lean insulin-sensitive individuals. Broader investigations of a role of AA concentrations in the context of various pathophysiological processes have coincided with the advent of metabolomics as a tool for studying human diseases (White and Newgard 2019). Metabolomics provides a snapshot of physiological/patophysiological processes (Zhao et al. 2017).

Since then, an overwhelming number of published data have repeatedly described several changes in metabolites, including increases in BCAA and other AA levels associated with visceral obesity (Yamakado et al. 2012) and IR (Palmer et al. 2015; Seibert et al. 2015; Nakamura et al. 2014; Nagao and Kimura 2020). A summary of current human studies reporting association of amino acids with insulin resistance and T2DM is presented in Table 1. Systemic BCAAs are strongly associated with IR and further T2DM development (Felig et al. 1969; Ferrannini et al. 2013; Wang et al. 2011; Würtz et al. 2013; Biswas et al. 2019). Moreover, numerous human studies have consistently demonstrated that concentrations of BCAAs in plasma as well as in urine have the quality to predict diabetes development (Yousri et al. 2015; Giesbertz and Daniel 2016). The correlation between IR and elevated circulating

Table 1 Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
(Gall et al. 2010)	2010	The RISC study (Relationship of Insulin Sensitivity to Cardio-vascular Risk), comprising a nondiabetic cohort	Nondiabetic subjects representing a broad spectrum of insulin sensitivity and glucose tolerance	399 subjects	fasting plasma	Nontargeted fashion on three separate mass spectrometry platforms, UHPLC-MS/MS (+/− ESI) and GC-MS (+EI)	α–hydroxybutyrate was shown as an early marker for both insulin resistance and impaired glucose regulation
(Fiehn et al. 2010)	2010		Overweight to obese T2DM and nondiabetic Gullah-speaking African-American women with or without a UCP3 g/a missense polymorphism	44 obese T2DM and 12 obese nondiabetic African-American women	Plasma	Gas chromatography–mass spectrometry	AA levels and their derivatives (i.e., Leu, 2-ketoisocaproate, Val, Cys, His) were increased significantly in T2DM subjects Leu and Val concentrations rose with increasing HbA1c, and significantly correlated with plasma acetylcarnitine concentrations
(Tai et al. 2010)	2010		Nonobese Asian-Indian and Chinese men from a large cross-sectional study carried out in Singapore	263	Plasma	MS-based metabolic profiling	Increased levels of Ala, Pro, Val, Leu/Ile, Phe, Tyr, Glu/Gln, and Orn, and a cluster of branched-chain and related amino acids were associated with IR. Increased abdominal adiposity and leptin, and decreased adiponectin and IGF-binding

(continued)

Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
							protein 1 were also correlated with IR
(Wang et al. 2011)	2011	Nested case-control study in the Framingham Offspring Study Prospective cohort Malmö Diet and Cancer study – replication cohort	Normoglycemic individuals	2422 (201 developed diabetes. during 12 years) metabolite profiling on the samples from 189 cases and 189 controls (mean age 57 years, 42% women) 163 cases and 163 controls (mean age 58 years, 55% women)	Plasma	Targeted approach using liquid chromatography with a triple quadrupole tandem mass spectrometry	Ile, Leu, Val, Tyr, and Phe had highly significant associations with future diabetes. The results were replicated in an independent, prospective cohort. These findings underscore the potential key role of amino acid metabolism early in the pathogenesis of diabetes and suggest that amino acid profiles could aid in diabetes risk assessment
(Cheng et al. 2012)	2012	2 nested case-control studies designed to investigate predictors of diabetes mellitus and cardiovascular disease	Individuals free of diabetes mellitus and cardiovascular disease	1761 individuals free of diabetes mellitus and cardiovascular disease at the original examination from two large, well-characterized	Plasma	LC-MS	Metabolic risk factors, such as obesity, insulin resistance, high blood pressure, dyslipidemia were associated with multiple metabolites including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown

				clinical cohorts in the Framingham Heart Study (FHS; $N = 1015$) and Malmö Diet and Cancer Study (MDC; N = 746). In MDC, a diagnosis of new-onset diabetes after the baseline examination (mean follow-up time 12.6 years)			products, and nucleotide metabolites. Moreover, a particularly strong association of insulin resistance traits with decreased Gln and increased Glu was observed. High glutamine-glutamate ratio was associated with lower risk of incident diabetes in FHS, but not in MDC
(Shah et al. 2012)	2012	Interventional	Nondiabetic individuals (37.4% African-Americans and 62.6% White) who had lost ≥4 kg during 6 months (phase I)	500	Plasma	Targeted mass spectrometry-based profiling	A cluster of metabolites comprising BCAA levels and related analytes predicted improvement in HOMA-IR independent of the amount of weight loss
(Stančáková et al. 2012)	2012	Population-based Metabolic Syndrome in Men (METSIM)	Nondiabetic or newly diagnosed type 2 diabetic Finnish men	9369 (4.7-year follow-up)	Plasma	Proton nuclear magnetic resonance spectroscopy	The levels of Leu, Ile, Tyr, and Ala increased and the levels of Gln and His decreased with increasing glycemia, reflecting, at least in part, insulin resistance (except for Gln) Only 1 of 43 risk single nucleotide polymorphisms

(continued)

Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
							regulating hyperglycemia, the glucose-increasing major C allele of rs780094 of GCKR, was significantly associated with decreased levels of Ala and Ile and elevated levels of Gln
(Wang-Sattler et al. 2012)	2012	Prospective cooperative Health Research in the Region of Augsburg (KORA) cohort Cross-sectional EPIC-Potsdam cohort – replication cohort	German individuals	4261 1297 cross-sectional KORA S4 study: 91 T2DM patients; 1206 individuals with non-T2DM (including 866 participants with NGT, 102 with i-IFG and 238 with IGT) 1010 prospective KORA S4→F4 study 876 non-T2DM individuals 91 incident cases of T2DM (mean follow-up 7 years) 641 NGT individuals	Serum	Liquid chromatography and flow injection analysis–mass spectrometry	Three metabolites (Gly, lysophosphatidylcholine (LPC) (18:2), and acetylcarnitine) had significantly altered levels in IGT individuals as compared to those with normal glucose tolerance. Lower levels of Gly and LPC were found to be predictors not only for IGT but also for T2DM, and were independently confirmed in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort

				118 incident IGT (mean follow-up 7 years)			
(Yamakado et al. 2012)	2012		Obese Japanese subjects	1449 (985 men and 464 women)	Plasma	LC-MS following derivatization	Accumulated visceral fat altered the peripheral amino acid profile. A multivariate logistic regression model of PFAAs could distinguish visceral obesity. This profile can be used as a predictor of elevated visceral obesity and a risk assessment tool for metabolic complications
(Magnusson et al. 2013)	2013	Matched case-control study derived from the population-based Malmö Diet and Cancer Cardio-vascular Cohort (MDC-CC)	Nested case-control study (MDC-CC) 4577 free of myocardial infarction or stroke (CVD) 253 incident CVD cases (during a mean follow-up time of 12.2 years) were matched with 253 controls. Cross-sectional study CVD case-control material from MDC-CC 506 (253 cases and 253 controls) and 564 free from prevalent CVD for cross-sectional analyses of DM-AA score in relation to intima-media thickness (IMT) of the common carotid artery (CCA), and classified using six-graded		plasma	LC-MS	Fasting plasma levels of Ile, Tyr and Phe were shown to predict diabetes development. The combination of these three AAs also predicted future cardiovascular events during long-term follow-up most likely through increased propensity of atherosclerosis BCAAs and AAAs were identified as novel markers of CVD development and as an early link between diabetes and CVD susceptibility

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
			plaque score Distinct cohort of subjects who underwent exercise stress testing with myocardial perfusion imaging: 83 cases with inducible ischemia and 83 control subjects				
(McCormack et al. 2013)	2013	Cross-sectional cohort and prospective longitudinal cohort (for 18 months)	Healthy individuals in Boston (21 African-Americans, 36 White, and 12 others) aged 8 –18 years. A subset of 17 individuals who were pre- or early-pubertal, aged 8–13 years, were enrolled in a prospective longitudinal cohort	69 (40 boys and 29 girls) – cross-sectional cohort 17 participants with a complete data – prospective longitudinal cohort	Plasma	Targeted LC-MS/MS-based profiling	Elevations in the concentrations of BCAAs were significantly associated with BMI in the cross-sectional cohort. In the subset followed in longitudinal study, baseline BCAA levels were positively associated with HOMA-IR measured 18 months later. Elevated BCAA levels were significantly associated with obesity in children and adolescents, and may independently predict future IR
(Würtz et al. 2013)	2013	Prospective cohort	Nondiabetic young Finnish adults from the	1680 (769 men and 911 women)	Fasting serum	High-throughput NMR spectroscopy	Circulating BCAAs (Ile, Leu, Val) and aromatic amino acids (Phe and Tyr) from fasting serum were shown as

			Cardiovascular Risk in Young Finns Study				predictors of the insulin resistance index, but not of glycemia, at 6-year follow-up in young, normoglycemic adults, with most pronounced associations for men
(Floegel et al. 2013)	2013	European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study Cooperative Health Research in the Region of Augsburg study – replication cohort (KORA) Tübingen Family study for T2DM (TüF)	German individuals TüF a family history of T2D, a BMI .27 kg/m^2, and previous impaired glucose tolerance or gestational diabetes mellitus	2282 randomly drawn (EPIC)-Potsdam subcohort (62.0% women) 800 (42.2% women) incident cases of T2DM (mean follow-up 7 years) and 876 KORA subcohort 91 incident cases of T2DM (mean follow-up 7 years) and 76 Caucasians TüF cohort	serum	Flow injection analysis tandem mass spectrometry (targeted metabolomics approach)	Phe was independently associated with increased risk of T2D and Gly was associated with decreased risk of T2DM
(Ferrannini et al. 2013)	2013	Two observational cohorts: the prospective, observational cohort study Relationship between Insulin	1,261 nondiabetic participants from the Relationship between Insulin Sensitivity and Cardiovascular Disease (RISC) study, with 3-year	1,261 and 2,580	Fasting plasma	Targeted LC-MS/MS-based profiling	α-hydroxybutyrate (α-HB) and linoleoyl-glycerophosphocholine (L-GPC) were identified as joint markers of insulin resistance (IR) and glucose intolerance. BCAAs; Leu, Ile, Val, and

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
		Sensitivity and Cardiovascular Disease (RISC) study and 2,580 from the family-based, observational familial Botnia Prospective Study	follow-up Subjects from 13 countries in Europe and 2,580 from the Botnia Prospective Study with 9.5-year follow-up Subjects from the West coast of Finland				three major glucogenic amino acids (Ala, Glu, Arg) were increased, whereas Gly was significantly decreased, in progressors versus nonprogressors. Increased concentrations of BCAAs and fatty acids, such as oleate, were positively related α-HB, whereas L-GPC and insulin sensitivity were reciprocally related to α-HB
(Wang et al. 2013)	2013	Nested case–control study from the Framingham Heart Study	Normoglycemic participants	2422 188 individuals who developed diabetes during 12 years and 188 propensity-matched controls	Plasma	Targeted LC-MS/MS-based profiling	The metabolite 2-aminoadipic acid (2-AAA) was most strongly associated with the risk of developing diabetes
(Mai et al. 2013)	2013	Part of a sample from a population from Eastern Germany, the Sorbs	German individuals with normal glucose tolerance, isolated impaired fasting glycemia, impaired glucose	1019 subjects Subjects with normal glucose tolerance (NGT; $n = 636$), isolated impaired fasting glycemia (IFG;	Serum	LC-MS	Alterations in serum concentrations of several acylcarnitines, in particular tetradecenoylcarnitine (C14:1), tetradecadienylcarnitine (C14:2), octadecenoylcarnitine (C18:

			tolerance or type 2 diabetes	$n = 184$), impaired glucose tolerance (IGT; $n = 87$), T2DM ($n = 112$)			1), and malonylcarnitine/ hydroxybutyrylcarnitine (C3DC+C4OH) are associated not only with T2D but also with prediabetic states
(Xie et al. 2014)	2014		Healthy obese and healthy lean participants (Chinese, $n = 105$ and American, $n = 72$)	106 healthy obese and 105 healthy lean participants	Serum	LC-MS and GC-MS	Metabolite profiles were significantly different between lean and obese participants. A cluster of obesity-associated changes in specific amino acids (BCAAs), fatty acids, acylcarnitine, and organic acid metabolites was identified in the obese participants but not in the lean participants. These metabolites were also associated with IR. Additionally, differences in serum metabolites and metabolic alterations, including BCAAs, fatty acids, UA, and creatinine, in obese humans occurred in a gender-dependent manner. Notably, three BCAAs (Ile, Leu, and Val) were correlated with IR and were differentially expressed

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
							in obese men, but not in obese women
(Nakamura et al. 2014)	2014	Cross-sectional cohort	T2DM Japanese subjects	51 (23 men and 28 women)	Plasma	LC-MS followed by precolumn derivatization	Glu, Tyr, Ala, Pro, and BCAAs were strongly correlated with the insulin-related variables such as C-peptide, insulin and HOMA-IR. Glu, Ala, Trp, and BCAAs were negatively correlated with adiponectin levels. The PFAA profiles in diabetic patients were strongly associated with hyperinsulinemia and hypoadiponectinemia, which might become risk evaluation factors for the development of cardiovascular diseases
(Roberts et al. 2014)	2014	Longitudinal, community-based Framingham Heart Study (FHS) HERITAGE		2067	Plasma	LC-MS	β-aminoisobutyric acid (BAIBA) plasma concentrations were inversely correlated with cardiometabolic risk factors (fasting glucose, insulin,

		Family Study, sedentary subjects were recruited for a 20-week program of supervised exercise training					HOMA-IR, triglycerides, and total cholesterol) in humans and were increased during exercise training
(Tillin et al. 2015)	2015	Cross-sectional and prospective study in South Asian and European cohort from the SABRE (Southall And Brent REvisited) Study	European and South Asian nondiabetic men	1,279 European and 1,007 South Asian nondiabetic men 801 European and 643 South Asian participants Diabetes developed in 227 (35%) South Asian and 113 (14%) European men. after 19 years of the follow-up period	Serum	Nuclear magnetic spectroscopy	Concentrations of Ile, Phe, Tyr, and Ala were significantly higher in South Asian men, while cross-sectional correlations of AAs with glycemia and insulin resistance were similar both in Europeans and South Asians. Stronger adverse associations were observed between branched chain and aromatic AAs, particularly tyrosine, and incident diabetes in South Asian men
(Seibert et al. 2015)	2015	Cross-sectional study	Nondiabetic individuals	182 (118 women and 64 men)	Plasma	LC-MS	14 out of 24 AA levels were significantly higher in males than females; the only Gly was lower in males. Glu, Ile, Leu, and Tyr levels had the strongest correlation with steady-state plasma glucose. This association was similar in women and men,

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
							independent of obesity, and similar to traditional markers of insulin resistance. In comparison to women, men tended to have a more unfavorable AA profile with higher AA levels associated with insulin resistance and less glycine. However, the degree of association between a direct measurement of insulin resistance and AA levels were similar between sexes and equivalent to several traditional markers of insulin resistance
(Allam-Ndoul et al. 2015)	2015		Overweight/obese subjects with or without metabolic syndrome (MetS) and normal weight subjects without MetS	200 (101 men and 99 women)	Fasting plasma	Mass spectrometry-based metabolite profiling	BCAAs were associated with obesity and MetS. Overweight/obese participants irrespective of their MetS status had higher plasma BCAA levels than normal weight participants. Obesity-associated MetS appeared to worsen the difference with normal weight subjects. Leu and Ile levels were correlated with HOMA-IR among obese individuals

							without MetS. Among obese subjects with MetS Leu levels were correlated to HOMA-IR whereas Leu and Ile were correlated with plasma glucose levels
(Yamakado et al. 2015)	2015		Japanese subjects	3701 Visceral fat area (VFA) and 2-h post-challenge insulin (Ins120 min) values were determined in 865 and 1,160 subjects, respectively. The cohort analysis was carried out, in which the individuals without these diseases were selected from a total of 2984 (1877 men) individuals (2,729 subjects without DM -, 91.5%; 2,695 individuals without metabolic syndrome, 90.3%; 2,336 without	Plasma	LC-MS followed by precolumn derivatization	BCAAs (Ile, Leu) and AAAs (Tyr, Phe) were statistically significantly related to the development of DM; Ile, Leu, Tyr, Ala, and Ser were significantly related to the development of metabolic syndrome; Ile, Leu, Tyr, Val, Ala, Pro, Ser, and Gly were significantly related to the development of dyslipidemia over the 4-year time period after adjusting for age, gender, BMI, fasting plasma glucose (FPG), and HOMA-IR. The PFAA models were able to predict the 4-year risk of developing lifestyle-related diseases, including DM, metabolic syndrome, dyslipidemia, and hypertension. The correlation coefficients of the obtained PFAA models

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Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
				dyslipidemia, 78.3%; or 2,637 without hypertension, 88.4%). Capabilities of the obtained models for predicting 4-year risk of developing new-onset lifestyle-related diseases were examined in a cohort study of 2,984 subjects			against VFA or Ins120 min were higher than single PFAA levels, suggesting their usefulness for future risk prediction
(Yousri et al. 2015)	2015	Cross-sectional case-control study	Individuals with diabetes and controls of Arab and Asian descent embedded in the Qatar Metabolomics Study on Diabetes (QMDiab)	188 T2DM individuals and 181 controls	saliva, blood plasma, and urine	LC-MS and GC-MS	Perturbations in the glycolysis pathway are reflected by increased pyruvate and lactate levels, and perturbations in Phe and Tyr metabolism have been also shown. Increased proteolysis with aminoaciduria is reflected by increased urinary BCAA and AAA levels. The presence of subclinical ketoacidosis in

							some patients is indicated by increased concentrations of 3-hyroxybutyrate and 3-hydroxyisobutyrate. Some of the established biomarkers were identified in more than one biofluid, such as 1,5-AG and 2-hydroxybutyrate. Of the 16 newly identified metabolite associations, many are in pathways that play a role in diabetes, including β-hydroxypyruvate (Gly, Ser, and Thr metabolism), 3-methoxytyrosine and 4-hydroxyphenylpyruvate (Phe and Tyr metabolism), 1,3-dihydroxyacetone (glycolysis pathway) as well as arabitol, gluconate, ribose, and xylonate (nucleotide and pentosemetabolism), thus linking these metabolites for the first time to diabetes
(Su et al. 2015)	2015	Cross-sectional study and longitudinal study		27 subjects (33 to 61 years old) Cross-sectional study that involved 9 lean (7 women and 2 men) and	Adipose tissue	GC-MS	Total adipose tissue mmBCFA content was ~30% lower in obese than lean subjects and increased by ~65% after weight loss in the RYGB group. Adipose tissue

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
				9 obese (7 women and 2 men) subjects Longitudinal study that involved 9 obese subjects (8 women and 1 man), who were studied before and 1 year after Roux-en-Y gastric bypass (RYGB) surgery			mmBCFA content correlated positively with skeletal muscle insulin sensitivity
(Chen et al. 2016)	2016	Cross-sectional and Longitudinal cohort study	Chinese participants at different stages of diabetes development	429 213 51 individuals (47% male) developed diabetes and 162 (27% male) remained free of diabetes after a median follow-up time of 10.0 years and 216 in the cross-sectional study	Serum	LC-MS	Early elevation of Val, Leu, Ile, Tyr, and Phe was closely associated with future development of diabetes, suggesting an important role of these metabolites as early markers of diabetes, highlighting the predictive value of these markers for future development of diabetes

(Palmer et al. 2015)	2015	Multiethnic cohort from the Insulin Resistance Atherosclerosis Study (IRAS)	European American, Hispanic, and African American nondiabetic at baseline subjects	196 72 high insulin sensitivity subjects and 75 Low insulin sensitivity subjects 146 76 converted to T2DM during a 5-year follow-up period and 70 nonconverters	plasma	Tandem mass spectrometry (MS/MS)	Gly was decreased and Val, Leu, Phe, and combined Gln and Glu were increased in insulin-resistant subjects. Ethnic-stratified results were strongest in European Americans. Comparing amino acid profiles between subjects that converted to T2DM yielded a similar pattern of associations: decreased Gly and increased Val, Leu, and combined Gln and Glu
(Lee et al. 2016)	2016		Nondiabetic participants of the Insulin Resistance Atherosclerosis Study (IRAS)	685 (290 Caucasians, 165 African Americans, and 230 Hispanics)	Plasma	Mass spectrometry	Plasma BCAAs were associated with incident diabetes and underlying metabolic abnormalities, although the associations were generally stronger in Caucasians and Hispanics
(Tricò et al. 2017)	2017	Cross-sectional and longitudinal study at the Yale Pediatric Obesity Clinic	Nondiabetic adolescents	78 nondiabetic adolescents 16 subjects after a mean follow-up of 2.3 years	Plasma	Nuclear magnetic resonance spectroscopy	BCAAs and α-hydroxybutyrate concentrations during an oral glucose tolerance test (OGTT) characterized insulin-resistant youth and predicted worsening of glycemic control

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
(Haufe et al. 2017)	2017	Prospective study	Overweight to obese individuals before and after 6 months on hypocaloric diets reduced in either carbohydrates or fat	109	Plasma (and urine)	GC-MS	3-HIB relates to insulin sensitivity but is not associated with intramyocellular fat content in overweight to obese individuals. Moreover, changes in 3-HIB rather than changes in BCAAs are associated with metabolic improvements with weight loss
(Harris et al. 2017)	2017		Sedentary, 50- to 65-year-old women with a stable weight basal conditions and during a hyperinsulinemic-euglycemic clamp procedure (HECP) with and without concomitant ingestion of protein ($n = 15$) or an amount of leucine that matched the	30	Plasma	GC-MS	The decrease in plasma 3-HIB concentration and increase in plasma FGF21 concentration induced by insulin and glucose infusion during hyperinsulinemic-euglycemic clamp procedure (HECP) is blocked by protein ingestion and the protein-induced increase in circulating 3-HIB and decrease in circulating FGF21 were associated with a marked impairment in insulin-stimulated glucose disposal. Val and FGF21 metabolism are involved in the pathogenesis of insulin resistance in skeletal muscle

			amount of protein ($n = 15$)				induced by protein ingestion in humans
(Shi et al. 2018)	2018	Nested case–control study within the Swedish cohort of the Västerbotten prospective population-based intervention program	Swedish incident diabetes cases and nondiabetic controls	503 participants at baseline who developed T2DM after a median time of 7 years. Among the 503 pairs of selected participants, 187 case-control pairs at 10-year follow-up	Plasma	LC-MS	Identified metabolites strongly correlated with insulin resistance and/or beta cell dysfunction. Changes in phosphatidylcholines (PCs) with odd-chain fatty acids, branched-chain amino acids, 3-methyl-2-oxovaleric acid, and glutamate over time along with disease progression among diabetes cases. Among other findings, PCs containing odd-chain fatty acids (C19:1 and C17:0) and 2-hydroxyethanesulfonate were associated with the likelihood of developing T2DM
(Flores-Guerrero et al. 2018)	2018	Prevention of renal and vascular end-stage disease (PREVEND) cohort	Residents from Groningen, the Netherlands	Prospective study 6244 subjects 301 cases of T2DM (during median follow-up for 7.5 years)	Plasma	Nuclear magnetic resonance spectroscopy	High BCAA levels were associated with insulin resistance and with increased risk of type 2 diabetes. This association was independent of multiple risk factors, HOMA-IR, and β cell function

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Table 1 (continued)

Associations between amino acids and insulin resistance and T2DM – summary of current studies mentioned in the respective sections							
Reference	Year	Type of study	Subjects	n	Type of material	Method	Main conclusions
(Yamakado 2018)	2018				Obtained model uses plasma AA levels, which correlate to visceral fat area, as an indicator of prediabetic visceral fat accumulation	AminoIndex LifeStyle diseases (AILS) test as a multivariate formula using plasma AA levels for lifestyle-related disease risk screening	Evidence is accumulating that the features of AILS include its ability to assess the risk for diabetes, suggesting it can be used for diabetes prediction
(Chen et al. 2019)	2019	Prospective nested case-control Hitachi Health Study	Nondiabetic Japanese working adults	4754 individuals 284 T2DM cases during a 5-year follow-up and 560 controls	Serum	LC-MS	High concentrations of Val, Leu, Ile, Phe, Tyr, Ala, Glu, Orn, and Lys were associated with an increased risk of incident T2DM. High Gln levels were associated with a decreased risk of incident T2DM. These AAs may be novel useful biomarkers in the identification of people at risk of T2DM before overt symptoms. Insulin resistance may account for or mediate the relationship between these AA and risk of incident T2DM

BCAA levels has been confirmed in several studies involving different ethnic groups and degrees of obesity (Newgard et al. 2009; Shah et al. 2012; McCormack et al. 2013; Würtz et al. 2013; Bi and Henry 2017). FAA profiles, particularly BCAA levels, are altered prior to the development of T2DM and are significantly associated with future diabetes diagnosis (Wang et al. 2011). These changes in plasma-free amino acids (PFAAs) can predominantly result from a metabolic shift caused by early diabetes pathogenesis (Nie et al. 2018) and may serve as a better indicator of impaired IR in prediabetic state than plasma glucose levels (Allam-Ndoul et al. 2015).

In addition, the dietary patterns of protein and BCAAs supplementation also significantly influence the association between BCAAs and IR (Zheng et al. 2016; Nagata et al. 2013; Biswas et al. 2019). Infusion of BCAAs or leucine in humans, as well as dietary intake of BCAAs, reportedly worsened insulin sensitivity (Zheng et al. 2016; Nagata et al. 2013; Harris et al. 2017; Shah et al. 2012; Biswas et al. 2019), while low BCAAs consumption has been correlated with improvement in metabolic health and alleviating IR (amelioration of IR) (Biswas et al. 2019). High dietary consumption of BCAAs is suggested to increase the risk of incident IR and may accelerate the progression of metabolic disorders, such as metabolic syndrome, and diabetes, and is not associated with the pancreatic β-cells dysfunction and hyperinsulinemia in adults. Higher total dietary BCAA intake was associated with an increased risk of T2DM in three prospective cohort studies (Nagata et al. 2013; Zheng et al. 2016; Nie et al. 2018). Moreover, these results were similar in Asian population (Nie et al. 2018).

The circulating BCAAs (valine, leucine, isoleucine) and AAAs (tyrosine and phenylalanine) have been identified as risk factors for the development of T2DM (Guasch-Ferré et al. 2016; Wang et al. 2011; Floegel et al. 2013; Palmer et al. 2015; Tillin et al. 2015; Chen et al. 2016; Stančáková et al. 2012; Wang-Sattler et al. 2012; Chen et al. 2019). Evidence concerning other amino acids is inconsistent (Chen et al. 2019). For example, an inverse correlation of glutamine with T2DM risk has been shown in some (Stančáková et al. 2012; Cheng et al., 2012) but not all of the performed studies (Floegel *et al.*, 2013; Tillin et al. 2015; Wang-Sattler et al. 2012). Glutamate was positively associated with the occurrence of T2DM in cohort studies of Finnish from the Botnia Prospective Study (Ferrannini et al. 2013) and American adults from the Framingham Heart Study (FHS) cohort (Cheng et al. 2012), as well as Swedish cohort of the Västerbotten prospective population-based intervention program (Shi et al. 2018), whereas relations of glutamate were nonsignificant in the Malmö Diet and Cancer Study (MDC) cohort free of diabetes at baseline Swedish individuals (Cheng et al. 2012).

Glycine was reported to be inversely associated with the incidence of T2DM in Germany and West coast of Finland (Floegel et al. 2013; Wang-Sattler et al. 2012; Ferrannini et al. 2013). It was also negatively correlated with incident diabetes in European but not in South Asian men (Tillin et al. 2015). Moreover, limited evidence suggests a positive association with T2DM in the case of alanine (Ala) and histidine as well as ornithine (Guasch-Ferré et al. 2016; Chen et al. 2019). Stronger correlation between BCAA concentrations and insulin sensitivity was observed in men, with

clear differences associated with age and ethnicity (Ianni et al. 2017). Consistent with this view, the population-based Cardiovascular Risk in Young Finns Study demonstrated that BCAAs, along with the aromatic amino acids: phenylalanine and tyrosine, were associated with IR selectively in men (Würtz et al. 2013). Xie et al. (2014) demonstrated that the serum metabolite profiles, verified with two independent groups of participants (Chinese, $n = 105$ and American, $n = 72$) of the obese population are gender-dependent. BCAA levels were correlated with IR and differentially expressed in obese men, but not in obese women (Xie et al. 2014). Another study found that 14 out of 24 measured AA levels were significantly higher in males than females; the only glycine was lower in males. Glutamic acid, isoleucine, leucine, and tyrosine levels had the strongest correlation with steady-state plasma glucose. This association was similar in women and men, independently of obesity, and similar to traditional markers of IR. In comparison to women, men tended to have a more unfavorable AA profile with higher AA levels associated with IR and less glycine. However, the degree of association between a direct measurement of IR and AA levels were similar between sexes and equivalent to several traditional markers of IR (Seibert et al. 2015).

The correlation between BCAA concentrations and T2DM development is also significantly modified by ethnicity, with the association in Caucasians and Hispanics while it does not appear in African Americans (Lee et al. 2016).

The cohort studies on the Asian population found BCAAs as a valid of the future risk of T2DM (Tillin et al. 2015; Tai et al. 2010), while another study of a predictive model in American Indians did not develop a reporter concept of BCAAs (Zhao et al. 2015).

Large, longitudinal studies confirmed that PFAAs analysis can predict the future susceptibility of lifestyle-related diseases (Wang et al. 2011; Yamakado et al. 2015), which is a significant strength of these investigations. Various prospective, case-controlled and nested studies on the subjects of different ethnic origins have shown elevated levels of BCAAs and other amino acids are associated with the prediabetic state, IR, and T2DM. Elevations in PFAA concentrations may independently predict the future development of diabetes, metabolic syndrome, dyslipidemia, or hypertension over a 4-year period, even after adjusting for commonly accepted risk factors such as age, sex, body mass index, fasting plasma glucose, IR, waist circumference, blood pressure, and lipid variables. From 2,984 Japanese subjects in the cohort study, 2,729 individuals without DM were included in the follow-up study to investigate the ability to predict the 4-year risk of developing new-onset DM. For single PFAA levels, BCAAs (isoleucine, leucine) and AAAs (tyrosine and phenylalanine) were significantly related to the development of DM over the 4-year time period after adjusting for age, gender, BMI, fasting plasma glucose (FPG), and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). Multiple linear regression analysis with variable selection models were constructed between PFAA levels and the visceral fat area (VFA) and 2-h post-challenge insulin (Ins120 min) values for predicting 4-year risk of developing new-onset lifestyle-related diseases. The correlation coefficients of the obtained PFAA models against VFA or Ins120 min were higher than single PFAA levels, suggesting their usefulness as versatile markers for health monitoring for future risk prediction (Yamakado et al. 2015).

The positive associations of BCAAs and aromatic amino acids with T2DM risk have subsequently been replicated in multiple other cohorts. Similarly, the Framingham Offspring Study, conducted among 2,422 normoglycemic individuals over a 12-year period, showed that BCAAs (isoleucine, leucine, valine) and AAAs (phenylalanine and tyrosine, and also tryptophan) levels have highly significant associations with future diabetes. The others were aromatic amino acids (tryptophan, phenylalanine, and tyrosine). A combination of three amino acids predicted future diabetes (with a more than fivefold higher risk for individuals in top quartile), highlighting the potentially crucial role of amino acid metabolism in early diabetes pathogenesis (Wang et al. 2011).

Chen and his colleagues verified the close correlation of valine, leucine, isoleucine, tyrosine, and phenylalanine with IR and the future development of diabetes in Chinese populations after 10 years of follow-up, suggesting an important role of these metabolites as early markers of diabetes (highlighting the predictive value of these markers for the future development of diabetes) (Chen et al. 2016).

Cheng et al. (2012) performed one of the most promising investigations to determine the plasma concentrations of 45 distinct metabolites and examine their relation to cardiometabolic risk among 1,761 individuals from two large, well-characterized clinical cohorts in the Framingham Heart Study (FHS; N=1015) and the Malmö Diet and Cancer Study (MDC; N=746). Metabolic risk factors, such as obesity, IR, high blood pressure, dyslipidemia were associated with multiple metabolites including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. Moreover, a particularly strong association of IR traits with decreased glutamine and increased glutamate was observed. High glutamine-glutamate ratio was associated with lower risk of incident diabetes in FHS, but not in MDC (Cheng et al. 2012). The prospective roles of circulating amino acids as reporter molecules of insulin sensitivity and diabetes were further investigated in 1,680 individuals from the population-based Cardiovascular Risk in Young Finns Study. Circulating BCAAs (isoleucine, leucine, valine) and aromatic amino acids (phenylalanine and tyrosine) from fasting serum were shown as predictors of the insulin resistance index, but not of glycemia, at 6-year follow-up in young, normoglycemic adults, with most pronounced associations for men. They were associated with HOMA-IR at baseline and for men at 6-year follow-up, while for women only leucine, valine, and phenylalanine predicted 6-year HOMA-IR ($P < 0.05$). These observations suggest that altered metabolism of BCAAs and AAAs precedes the development of IR in early adulthood, before the onset of impaired fasting glucose levels, which at least partially explains how these amino acids are associated with the risk of future type 2 diabetes (Würtz et al. 2013).

Tillin et al. performed the cross-sectional and prospective analyses of ethnicity, amino acids level, and diabetes in a South Asian and European cohort from the SABRE (Southall And Brent REvisited) study. The study was performed on 801 European and 643 South Asian participants with 19 years follow-up period. The authors concluded that branched-chain and aromatic amino acids may contribute to excess risk of diabetes development (Tillin et al. 2015).

Stančáková et al. investigated amino acid levels with proton nuclear magnetic resonance spectroscopy in the population-based Metabolic Syndrome in Men (METSIM) Study, including 9,369 nondiabetic or newly diagnosed T2DM Finnish men. The levels of leucine, isoleucine, tyrosine, and alanine increased, and the levels of glutamine and histidine decreased with increasing glycemia, reflecting, at least in part, insulin resistance (Stančáková et al. 2012).

In a prospective nested case-control study, conducted among Japanese employees during a 5-year follow-up, fasting serum concentrations of several amino acids, including valine, leucine, isoleucine, phenylalanine, tyrosine, alanine, glutamate, ornithine, and lysine were associated with an increased risk of incident T2DM. High glutamine levels were associated with a decreased risk of incident T2DM (Chen et al. 2019).

In another study performed among Chinese population, GC-MS-based metabolic profiling showed increased levels of several amino acids, involving BCAAs (leucine, isoleucine) as well as alanine, and serine while substantial lower levels of 2-ketoisocaproic acid as early biomarkers of T2DM. Lower concentrations of 2-ketoisocaproic acid, a product of leucine deamination, may indicate a reduced rate of conversion of leucine to 2-ketoisocaproic acid in T2DM (Zeng et al. 2009). Similarly, Fiehn et al. observed an increase in certain AA levels and their derivatives (i.e., leucine, 2-ketoisocaproate, valine, cystine, and histidine). It is noteworthly, that plasma leucine concentration was significantly increased by ∼50%, and its initial catabolic metabolite, 2-ketoisocaproic acid (α-ketoisocaproate), was significantly increased by ∼27%. Mean plasma valine level was ∼20% higher in T2DM subjects vs. nondiabetic weight/age matched African-American women, but this difference was not statistically significant. In addition, leucine and valine concentrations rose with increasing HbA1c, and significantly correlated with plasma acetylcarnitine concentrations (Fiehn et al. 2010).

Several other studies have observed the predictive ability of PFAA analysis in evaluating the risk of developing lifestyle-related diseases and associated cardiovascular diseases. Magnusson et al. (2013) investigated the metabolite profiles among 4577 subjects among whom in case of 253 first-incident of cardiovascular disease (CVD) (myocardial infarction or stroke) occurred during a mean follow-up time of 12.2 years. Fasting plasma levels of isoleucine, tyrosine, and phenylalanine were shown to predict diabetes development with a four- to sixfold increased risk for participants in the top quartile. The combination of these three amino acids may also predict future cardiovascular events during long-term follow-up. McCormack et al. reported that elevations in BCAA concentrations are significantly associated with obesity in children and adolescents, and may independently predict future IR. Elevated BCAA levels significantly correlated with BMI in the cross-sectional cohort. In the subset of participants followed longitudinally, baseline BCAA levels were positively associated with HOMA-IR measured 18 months later (McCormack et al. 2013). Metabolic profiling performed in baseline and after 6 months in plasma samples from 500 participants after at least 4 kg of weight loss in phase I revealed a cluster of metabolites comprising BCAA levels and related analytes that could predict improvement in HOMA-IR independently of the amount of weight lost

(Shah et al. 2012). Nakamura et al. (2014) recruited 51 Japanese subjects diagnosed with T2DM and measured their PFAA profiles. Several amino acids: BCAAs, glutamate, tyrosine, alanine, and proline were strongly correlated with the insulin-related variables such as C-peptide, insulin, and HOMA-IR. They also observed that the levels of BCAAs, glutamate, alanine, and tryptophan were negatively correlated with adiponectin concentrations. Adiponectin plays a pivotal role in the regulation of insulin sensitivity and metabolism. Adiponectin concentrations have shown to be decreased in obese people or diabetic patients and are strongly related to IR and hyperinsulinemia in humans (Ziemke and Mantzoros 2010). These results indicated the significant relationship between PFAA profiles, adiponectin levels, and IR (Nakamura et al. 2014).

A cross-sectional and longitudinal study conducted at the Yale Pediatric Obesity Clinic showed that BCAA and α-hydroxybutyrate concentrations during an oral glucose tolerance test (OGTT) characterize insulin-resistant youth and predict worsening of glycemic control (Tricò et al. 2017). Furthermore, in a nested case-control study within the Swedish cohort of the Västerbotten prospective population-based intervention program, an untargeted metabolomics of plasma samples from 503 case-control pairs at baseline (median time 7 years before diagnosis) and samples from a subset of 187 case-control pairs at 10-year follow-up was performed. As a result 46 metabolites allowing T2DM prediction were reported (Shi *et al.*, 2018). Identified metabolites strongly correlated with IR and/or beta cell dysfunction. Among diabetes cases, changes in phosphatidylcholines (PCs) with odd-chain fatty acids, branched-chain amino acids, 3-methyl-2-oxovaleric acid, and glutamate were observed over time along with disease progression. Prospective associations between plasma BCAA levels and T2DM risk were also established in a population-based Prevention of renal and vascular end-stage disease (PREVEND) cohort. BCAA concentrations were determined by nuclear magnetic resonance spectroscopy in 6244 subjects, among whom 301 cases of T2DM were ascertained during a mean follow-up period of 7.5 years. High levels of BCAAs were confirmed as previously identified predictive biomarkers of IR and T2DM. This association was independent of multiple risk factors, HOMA-IR and β cell function (Flores-Guerrero et al. 2018)

The association between the level of circulating BCAAs, insulin resistant obesity, and T2DM prompted consideration of BCAA levels as a predictor for future IR or T2DM in order to develop screening PFAA-based tests. Analysis of general health check-up data from 8070 subjects revealed that the amino acid balance of precipitants that developed diabetes within 4 years was similar to that of individuals with diabetes, suggesting that changes in amino acid metabolism may occur prior to the onset of diabetes. Based on these findings, an index known as the AminoIndex LifeStyle diseases (AILS) test was developed. The obtained model was created as a multivariate formula using plasma AA levels, which correlate to visceral fat area as an indicator of prediabetic visceral fat accumulation. Plasma AA levels of asparagine, glycine, alanine, valine, tyrosine, and tryptophan levels were included in the AILS (risk of diabetes) formula derived in this study. Alanine, valine, tyrosine, and tryptophan levels were significantly higher in individuals who developed diabetes within 4 years compared with levels in those who were not diagnosed with diabetes during this period,

while levels of glycine were significantly lower. The AILS test was investigated whether its values normalize with such interventions as dietary and exercise counseling for 3 months. AILS values decreased significantly in individuals who managed to reduce body weight and waist circumference (Tochikubo et al. 2016), suggesting that early risk assessment using AILS could support early interventions in at-risk populations (Nagao and Kimura 2020). After commercialization in 2011, tests based on PFAAs were adopted in over 1500 clinics and hospitals in Japan, and numerous clinician-led studies have been performed to validate these tests. Evidence is accumulating that the features of AILS include its ability to assess the risk for diabetes, suggesting it can be used for diabetes prediction (Yamakado 2018).

Amino Acids Metabolic Intermediates As Markers for Insulin Resistance and Diabetes

Many recent studies suggest that not only BCAAs, but also BCAA catabolic enzymes and metabolic intermediates may play a key role in determining the relationship between BCAAs and other amino acids, and IR (Biswas et al. 2019). Obesity-related increase in BCAA levels may be the result of changes in amino acid metabolism, particularly decreased rates of their oxidation in adipose tissue (She et al. 2007; Lackey et al. 2013; Nagao and Kimura 2020; White and Newgard 2019). Recent metabolomics studies on diabetes have identified changes in plasma concentrations of BCAA-derived branched-chain keto acids, short branched-chain fatty acids, and various acylcarnitines as new entities with predictive qualities (Giesbertz and Daniel 2016).

Altered levels of BCAA-catabolic intermediates was shown to influence metabolic maladaptations during obesity and IR (Gall et al. 2010). Circulating BCKA and its association with heightened fasting plasma glucose and IR have been established by the Relationship of Insulin Sensitivity to Cardiovascular Risk (RISC) study group (Gall et al. 2010). Elevated plasma BCKAs were also confirmed by a few studies on mice (Lian et al. 2015).

Other intermediates related to BCAA catabolism are long-chain monomethyl and odd-numbered fatty acids. They can be derived from an odd-chain starter CoA with chain elongation in the fatty acid synthase complex. Monomethyl branched-chain fatty acids (mmBCFA) content in adipose tissue was reduced in obese compared to lean subjects, increased after weight loss in obese individuals, and correlated positively with insulin sensitivity (Su et al. 2015).

Val-derived metabolite, 3-hydroxyisobutyrate (3-HIB), is produced in skeletal muscle in response to forced expression of Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). 3-HIB, secreted from muscle cells is shown to regulate trans-endothelial fatty acid transport (White and Newgard 2019). Circulating 3-HIB is positively associated with blood glucose levels in diabetic patients, suggesting that this metabolite may promote excessive lipid accumulation and impaired insulin action in skeletal muscle (White and Newgard 2019). In overweight and obese subjects, lower circulating 3-HIB levels, but not BCAA, were associated

with metabolic improvement after weight loss (Haufe et al. 2017; Biswas et al. 2019) and were also correlated with insulin-stimulated glucose utilization in older obese women (Harris et al. 2017; Biswas et al. 2019). These results indicate that 3-HIB may serve as a signaling metabolite in IR and T2DM.

Interestingly, elevated plasma levels of β-aminoisobutyric acid (BAIBA) due to enhanced valine catabolism have also been shown to increase with exercise and are inversely correlated with such metabolic risk factors as fasting glucose, insulin, HOMA-IR, triglycerides, and total cholesterol in a randomized large human cohort enrolled in the longitudinal Framingham Heart Study (Roberts et al. 2014). BAIBA is identified as a novel small molecule myokine that increases brown adipocyte-specific genes expression in white adipose tissue and fatty acid β-oxidation in hepatocytes, both in vitro and in vivo, through a PPARα-mediated mechanism. Moreover it was shown to induce a brown adipose-like phenotype in human pluripotent stem cells, and improve glucose homeostasis in mice (Roberts et al. 2014; Biswas et al. 2019). Thus, BAIBA may contribute to exercise-induced protection against metabolic diseases (Roberts et al. 2014).

Newgard et al. showed that BCAAs-derived odd numbered 3- and 5-carbon acylcarnitines have a predictive value for the development of diabetes (Newgard et al. 2009). Acylcarnitines present in plasma and urine may reflect defective BCAAs mitochondrial oxidation. Various acylcarnitines derived from BCAA catabolism may be associated with IR and T2DM (Giesbertz and Daniel 2016). Mai et al. reported that alterations in serum levels of several acylcarnitines, in particular tetradecenoylcarnitine (C14:1), tetradecadienylcarnitine (C14:2), octadecenoylcarnitine (C18:1), and malonylcarnitine/hydroxybutyrylcarnitine (C3DC+C4OH) are correlated not only with T2DM but also with prediabetic states (Mai et al. 2013).

Lysine degradation product, 2-aminoadipic acid (2-AAA) was most strongly associated with the risk of diabetes development in the Framingham Heart Study (Wang et al. 2013). Participants with 2-AAA levels in the top quartile had greater than a fourfold risk of developing diabetes during the mean 12 years follow-up period. Administration of 2-AAA led to a reduction in fasting plasma glucose levels in mice fed both the standard chow and high fat diets. Moreover, treatment with 2-AAA increased insulin secretion from the pancreatic β cell line as well as murine and human islets. Our results suggest that 2-AAA is a diabetes risk marker and a potential modulator of glucose homeostasis in humans (Wang et al. 2013; Chen and Gerszten 2020).

A disadvantage of these new reporter molecules compared to BCAAs is the lower plasma and tissue levels of several species, resulting in higher analytical variation, with some compounds also less stable (Giesbertz and Daniel 2016).

Conclusions

Beyond their contribution as fundamental building blocks of life, amino acids play a key role in various physiological as well as pathological processes. Recent evidences demonstrate that elevated amino acids, especially BCAA levels, are associated with a number of pathologies, including: obesity, IR, T2DM, and CVD. Therefore, the

measurement and monitoring of circulating AA levels in biological fluids could represent a promising method for early detection of the disease and may provide a means of preventing its development. This chapter reviews the latest findings regarding the use of plasma/serum FAAs profiles as novel useful biomarkers in the identification of people at risk of T2DM before overt symptoms as well as focuses on potential targets relating to their signaling pathways, and metabolism that broadens our understanding of their role in insulin resistance and diabetes.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter changes in circulating AAs levels and their correlations with IR, prediabetic state, and T2DM have been reviewed. Several studies have been explored in order to identify and integrate AAs metabolite biomarkers proposed so far. A large body of evidence shows that AAs, especially BCAAs, are associated with obesity, IR, prediabetic state, and T2DM as well other pathological conditions, including cancers, CVD, liver disease, chronic kidney disease (CVD), and ischemic stroke, highlighting the potential use of AAs profiling as a valuable diagnostic tool to access the risk of disease development. The use of AAs as novel disease biomarkers in clinical practice may improve treatment strategies.

Mini Dictionary of Terms

- **HOMA-IR** – Homeostatic Model Assessment (HOMA) is a method for assessing β-cell function and insulin IR from basal (fasting) glucose and insulin or C-peptide concentrations. It approximates insulin resistance.
- **INSULIN RESISTANCE** – A pathological condition in which cells fail to respond normally to the insulin hormone.
- **BIOMARKER** – A measurable biological feature that can distinguish normal condition from pathological condition or indicate a response to an administered therapeutic drug.
- **METABOLOMICS** – A scientific discipline devoted to study changes in metabolome by measurement of small molecule metabolites. Nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS), coupled with liquid chromatography (LC-MS), gas chromatography (GC-MS), or capillary electrophoresis (CE-MS), is usually used to measure metabolites. Not only primary metabolites, but also the substrates and products of metabolism are measured. Metabolites can be measured in a targeted (metabolic profiling) or an untargeted (metabolic fingerprinting) manner.
- **T2DM** – Most commonly diagnosed type of diabetes, characterized by abnormal glucose and lipid metabolism, resulting from resistance to the effects of insulin and insufficient response to the secretion of this hormone. As this disease progresses, there may also occur a partial β-cell insufficiency and deficiency in insulin production.

Key Facts of Amino acids

A number of studies suggest that BCAAs supplementation or BCAA-rich diets has potential benefits for promoting lean body mass in obesity or catabolic disorders. Elevated BCAAs levels have been reported to improve body composition, glycemia levels, and to increase satiety for weight loss (Lynch and Adams 2014).

- In contrast to the potential health-promoting effects of BCAAs under conditions of negative energy balance, higher BCAAs intake may lead to adverse effects on the development of IR (Nie et al. 2018). Increased levels of branched-chain amino acids in plasma, serum, and also in urine are associated with obesity, IR, and diabetes, in particular T2DM.
- Branched-chain amino acids: valine, leucine, isoleucine, and aromatic amino acids, that is tyrosine and phenylalanine, have been demonstrated as the most consistent metabolite biomarkers for diabetes, in particular T2DM.
- Elevated BCAA levels have often been shown to predict the development of T2DM well in advance of its actual occurrence, what is very interesting from a diagnostics standpoint.
- BCAAs may not only have a "reporter quality" but may also contribute to the development of IR and T2DM.

Summary Points

- In the current medical situation, DM is a worldwide epidemic. Diabetes, particularly type 2 portends a poor prognosis and shortens the life expectancy of diabetic patients. Moreover, it imposes a heavy financial strain on health care systems everywhere.
- Early screening and testing of people at risk is the best approach to control the increasing numbers of diabetes occurrences, and it is most effective to recognize the early stages of DM before major systematic damage occurs.
- Various prospective, case-controlled and nested studies on the subjects of different ethnic origins shown that elevated levels of BCAAs and other AAs are associated with the prediabetic state, IR, and T2DM.
- Amino acids quantification in biological material, primarily in plasma, could be a valuable prognostic tool for determining metabolic abnormalities leading to diabetes.
- The activation of mTORC1 by BCAAs has been suggested to trigger IR, and subsequent metabolic disorders.
- Deficiencies in BCAAs metabolism referred as dysmetabolism lead to increased BCAA levels in obesity and/or diabetes. It can also induce the accumulation of possibly toxic intermediates, such as branched-chain keto acids, branched-chain fatty acids, and various acylcarnitines that impair cellular function(s) and may induce IR.
- Various intermediates of BCAAs are now considered as predictive markers that reflect IR and diabetes development.

The second possible mechanism is referred as BCAAs . Deficiency in BCAAs metabolism may induce the accumulation of toxic intermediates, such as branched-chain keto acids, branched-chain fatty acids, and various acylcarnitines that promotes cellular dysfunction, which leads to poorer insulin sensitivity of skeletal muscles.

Accumulation of BCAA break-down products may also interfere with proper mitochondrial function. Overloading of mitochondria with lipid substrates leads to oxidative stress and impaired insulin action contributing to insulin resistance.

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Components of the Purine Metabolism Pathways as Biomarkers for the Early Diagnosis of Diabetes

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Contents

Introduction 130
Purine Metabolism 131
Xanthine Oxidase 133
Adenosine Metabolizing Enzymes, Inosine and Adenosine 134
Inosine-5′-Monophosphate Dehydrogenase 139
Uric Acid 141
Conclusion 143
Applications to Prognosis, Other Diseases or Conditions 143
Mini-Dictionary of Terms 146
Key Facts of Purine Enzymes and Related Metabolites 146
Summary Points 147
References 147

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Abstract

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by persistent hyperglycemia and often hyperlipidemia. T2DM is usually accompanied with obesity, insulin resistance (IR), inflammation, and oxidative stress. Development of T2DM usually starts from IR and metabolic impairment (prediabetes), which may exist for years before the clinical manifestation of the disease and T2DM diagnosis.

Purinosome is an intricate system composed of numerous transporters, receptors, mediators, and catabolic and synthetic enzymes involved in the de novo biosynthesis of purines. This biological system is linked with glucose and lipid metabolic pathways. It is believed that purinosome system enzymes and metabolites regulate insulin secretion and glucose metabolism and consequently are involved in the pathological mechanisms of T2DM development. Numerous studies have reported altered levels of purinsome-based metabolites and enzymes or their catabolic products in obese, prediabetic, and metabolic syndrome subjects. These components of the purine metabolism pathways can be considered early biomarkers for the screening, early diagnosis, and monitoring of diabetes and related complications. Some of them (e.g., isopentenyladenosine-5-monophosphate, uric acid, xanthine, and xanthine oxidase) were reported as T2DM predictive biomarkers. The altered level of key purine enzymes and related metabolites in relation to T2DM, prediabetes, and obesity is discussed in this chapter.

Keywords

Diabetes · Prediabetes · Metabolic diseases · Insulin resistance · Obesity · Purine metabolism · Xanthine oxidase · Adenosine · Inosine · Uric acid · Biomarkers

Abbreviations

5′-NT	5′-nucleotidase
5-PRPP	5-Phosphoribosyl-1-pyrophosphate
ADA	Adenosine deaminase
Adok	Adenosine kinase
ALT	Alanine transaminase
AMP	Adenosine 5-monophosphate
AMPD	AMP deaminase
ApoB	Apolipoprotein B
APRT	Adenine phosphoribosyltransferase
AST	Aspartate transaminase
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
cAMP	Cyclic AMP
Cat	Catalase

CBS	Cystathionine beta synthase
CMP	Cytidine 5′-monophosphate
CVD	cardiovascular disease
CYP1A2	cytochrome P450 1A2
DBP	Diastolic blood pressure
DN	Diabetic nephropathy
DR	Diabetic retinopathy
Ecto5′NTase	Ecto-5′-nucleotidase
eGFR	Estimated Glomerular Filtration Rate
FAD	*Flavin adenine dinucleotide*
FAICAR	5-formamidoimidazole-4-carboxamide ribotide
FBG	Fasting blood glucose
FFA	Free fatty acids
GGT	γ-glutamyltransferase
GIP	Glucose-dependent insulin tropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT-4	Glucose transporter type 4
GMP	Guanosine-5-MP
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTP	Gamma-glutamyl transpeptidase
Gua	Guasine
Guo	Guanine
Hb1Ac	Glycated hemoglobin
HDL-C	High-density lipoprotein cholesterol
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HIF	Hypoxia-inducible factor
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
Hs-CRP	High-sensitivity C-reactive protein
IL-6	interleukin 6
IMP	Inosine monophosphate
IMPDH	IMP dehydrogenase
Inok	Inosine kinase
IR	Insulin resistance
IRS1	Insulin receptor 1
LDL-C	Low-density lipoprotein cholesterol
MCP-1	Monocyte Chemoattractant Protein-1
MDA	Malondialdehyde
MetS	Metabolic syndrome
NAD+	Nicotinamide adenine dinucleotide
NAT2	N-acetyltransferase 2
NO	Nitric oxide
OGTT	Oral *Glucose Tolerance Test*
PKB	Protein kinase-B
PNP	Purine nucleotide phosphorylase

PPAR-γ	Peroxisome proliferator-activated receptor gamma
R1P	Ribose 1-phosphate
R5P	Ribose 5-phosphate
RCT	Randomized controlled trial
SBP	Systolic blood pressure
SNP	Single-nucleotide polymorphisms
SOD	Superoxide dismutase
SUA	Serum uric acid
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
UA	Uric acid
UMP	Uridine 5′-monophosphate
WC	Waist circumference
XMP	Xanthosine 5′-monophosphate
XO	Xanthine oxidase

Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by persistent hyperglycemic and often hyperlipidemic states that can also be associated with other health issues like insulin resistance (IR), inflammation, obesity, dyslipidemia, oxidative stress, etc. These states usually lead to other related severe health conditions (e.g., diabetic retinopathy, kidney disease, and diabetic ketoacidosis) and usually start from undiagnosed IR and metabolic impairment existing for years (Hameed et al. 2020a). This transition from metabolic impairment (prediabetes) to diabetes may take years and usually occurs before the clinical manifestation of the disease. Knowledge of molecular mechanisms occurring during this transition could be a key to understanding the pathophysiology of T2DM. Moreover, at this early stage, the process can be reversed. Therefore, biomarkers for the early diagnosis of metabolic impairment are needed (Hameed et al. 2020b).

Purinosome is an intricate system composed of numerous transporters, receptors, mediators, and catabolic and synthetic enzymes involved in the de novo biosynthesis of purines (Antonioli et al. 2019). This biological system directly governs the metabolism of hexoses and lipids by keeping the normal concentrations of its signaling metabolites (Concepcion et al. 2020). The systematic linkage of purinosome with glucose and lipid metabolic pathways is discussed below. The concentrations of purinosome metabolites are tightly monitored by purinosome enzymes, which are also believed to regulate insulin secretion and glucose metabolism (Romeo and Jain 2020). It is also suggested that they are involved in the pathological mechanisms of T2DM development. Numerous studies have reported altered levels of purinsome-based metabolites and/or their catabolic products in obese, prediabetic, and metabolic syndrome subjects in comparison to healthy

controls (Varadaiah et al. 2019). These metabolites can be considered early biomarkers for the screening, early diagnosis, and monitoring of diabetes and related complications. A large and diverse amount of data has been published in this regard, which is collected and critically summarized in this chapter.

Purine Metabolism

Purines are a fundamental part of nucleotides and nucleic acids involved in a myriad of cellular processes and pathways like energy homeostasis (storage and transfer of ATP), transportation of biological groups (glycosyl, alcohol phosphate, sulfate, hydrogen, hydride ion, electrons, and alkyl group) or regulation of many enzymes of catabolic pathways through the allosteric effects. They are also building blocks for DNA and RNA as well as structural units of significant signaling coenzymes (NAD+, FAD, or CoA) and low-molecular-weight compounds (Pedley and Benkovic 2017). As purines and related metabolites are involved in all forms of life, keeping their optimum cellular level is of great importance. Their tightly regulated biosynthesis and degradation assure it. Purines are produced in the liver by highly conserved two pathways, i.e., de novo biosynthesis and complementary salvage pathway (Fig. 1). Their de novo biosynthesis is a multistep process that uses the substrates (i.e., ATP, CO_2, glycine) coming from energy homeostasis and the pentose-phosphate pathway. The salvage pathway relies on the catabolic products of purines. Under healthy conditions, the salvage pathway exerts feedback control over the de novo biosynthesis (Zhao et al. 2015). In mammals, de novo biosynthesis is primarily responsible for fulfilling the basic purine needs and replenishing their pool during cell proliferation. It is an energy-intensive and highly conserved ten-step enzymatic process that associates itself with energy-producing pathways by using their substrates (Zhang et al. 2008). As it can be seen in Fig. 1, in the first step, ribose-5-phosphate coming from the pentose-5-phosphate pathway is transformed to 5-phosphoribosyl-1-pyrophosphate (PRPP) through the action of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthase. Further addition of numerous amino acid substrates (glutamine, glycine, formate, and aspartate) and one carbon unit contribute to making inosine monophosphate (IMP) (Zhang et al. 2008). It is estimated that five ATP, two molecules of glutamine and formate, and one molecule of glycine, aspartate, and carbon dioxide are necessary to generate one IMP molecule. IMP is further down-processed initially into adenosine-5-MP (AMP) or guanosine-5-MP (GMP), which are also converted into related di- and tri-nucleotides, deoxyribonucleotides, and eventually incorporated into RNA and DNA. The complementary salvage pathway is mainly composed of superior end-products of the de novo biosynthesis pathway and is involved in the interconversions of hypoxanthine, nucleotides, and di-nucleotides to their monophosphate forms. In salvage pathway, guanine, hypoxanthine and adenine are converted to mononucleotides by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and adenine phosphoribosyltransferase (APRT), while deoxyguanosine and adenosine are functionally activated by enzyme-specific kinases (Yamaoka et al. 1997). In addition to the

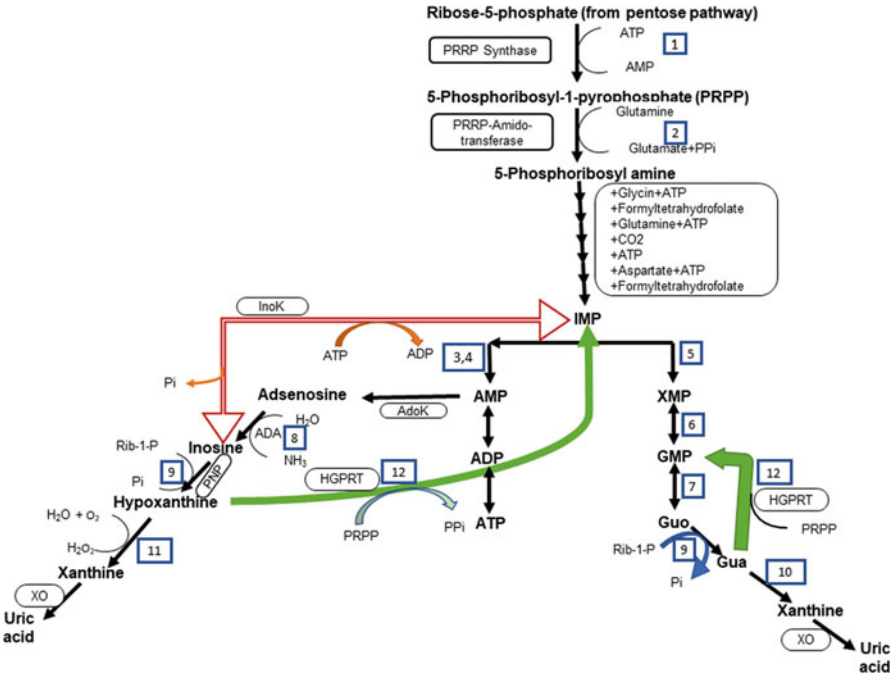

Fig. 1. A schematic representation of purine metabolism (de novo biosynthesis, degradation, and salvage pathway) in which key reactions from 5-PRPP synthase to the final degradation into uric acid are sequentially presented. Purine Biosynthesis: [1] 5-Phosphoribosyl-1-pyrophosphate (PRPP) synthesis is catalyzed by PRPP synthetase. Note: the ribose-5-phosphate for the pathway comes from the Pentose Phosphate Pathway. [2] This step is regulated and catalyzed by PRPP amidotransferase. Glutamine offers the nitrogen to start purine synthesis. Inosinic acid (IMP) is the first purine formed, in total six high energy phosphate bonds are consumed to produce IMP. [3] IMP + Aspartate + GTP --- > Adenylosuccinate; Enzyme: Adenylosuccinate Synthetase. [4] Adenylosuccinate --- > AMP + Fumarate; Enzyme: Adenylosuccinase. [5] IMP + NAD^+ --- > XMP + NADH + H^+; Enzyme: IMP Dehydrogenase. [6] XMP + Glutamine + ATP --- > GMP + Glutamate + AMP + PP_i. Purine degradation. [7] Nucleotidase: Purine Mononucleotide + H_2O --- > Purine Nucleoside + P_i. [8] Deaminase: Adenosine + H2O --- > Inosine + NH_4^+. [9] Purine Nucleoside Phosphorylase: Inosine + P_i --- > Hypoxanthine + R-1-P; Guanosine + P_i --- > Guanine + R-1-P; Note: R-1-P can be converted to R-5-P. [10] Guanase deaminates guanine to xanthine. [11] Xanthine Oxidase oxidizes hypoxanthine to xanthine, then xanthine to uric acid, which is excreted in the urine. Co-factors: Mo, Fe^{3+}, FAD, O_2. Note: H_2O_2, a product of this reaction must be scavenged by Glutathione Reduced. Salvage pathway: [12] Purines can be salvaged after steps [7] and [8] by reconverting the nucleosides to nucleotides via nucleotide kinases. The free base can also be saved in a salvage pathway consisting of a single enzyme, HGPRT: Hypoxanthine + PRPP --- > IMP + PP_i; Guanine + PRPP --- > GMP + PP_i. The importance of this pathway as a source of nucleotides is shown by the consequences of a deficiency of HGPRT known as Lesch-Nyhan syndrome. This pathway reduces the need and energy expenditure of biosynthesis by maintaining nucleotide levels. R1P, ribose 1-phosphate; R5P, ribose 5-phosphate; AdoK, adenosine kinase; ADA, adenosine deaminase; PRPP, 5-phosphoribosyl-1-pyrophosphate; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; XO, xanthine oxidase; IMP, inosine monophosphate; InoK, Inosine Kinase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; AMP, Adenosine 5′-monophosphate; XMP, xanthinin 5′-monophosphate; GMP, guanosine 5′-monophosphate; CMP, Cytidine 5′-monophosphate; Guo, guanine; Gua, guasine; UMP, Uridine 5′-monophosphate; PNP, purine nucleotide phosphorylase

salvage pathway, as mentioned above, another mechanism also runs in parallel to maintain the optimum cellular level of purines called the catabolic pathway of purines (Cohen A Fau - Barankiewicz and Barankiewicz). In this pathway, adenine and/or guanine are phosphorylated and deaminated rapidly into inosine (Fig. 1). Subsequently, inosine is converted to hypoxanthine, which is converted to xanthine with xanthine oxidase (XO). Xanthine also acts as a substrate for XO and enhances superoxide generation (Burnstock and Novak 2013). In metabolic syndrome, this superoxide damages tissues causing microvascular dysfunctions. Finally, xanthine is also converted into the final product of purine catabolism, uric acid (UA). UA is not only a biomarker of unhealthy metabolism but is also considered a true mediator of microvascular and macrovascular, i.e., related to diabetes, complications. Blood UA levels are well regulated in healthy persons whereas its increase is observed in many metabolic disorders. Hyperuricemia is a consequent of perturbation in purine catabolism, which also disturbs the level of blood glucose and other purine degradation products, i.e., xanthine, hypoxanthine, allantoin, inosine, and adenine. It has been reported (Drogan et al. 2015) that purines/pyrimidines and deoxy-sugars are relatively more reliable T2DM risk predictors than phospholipids/glycerophospholipids. Some of them (e.g., isopentenyladenosine-5-monophosphate, UA, XO, xanthine) were found as T2DM predictive biomarkers. The altered level of key purine enzymes and their metabolites in relation to T2DM are discussed in detail below.

Xanthine Oxidase

XO is a metalloflavoenzyme existing in two interconvertible forms, i.e., dehydrogenase and oxidoreductase. Both forms catalyze the formation of UA. However, pathophysiological conditions (i.e., hyperglycemia, hyperlipidemia, oxidative stress, hypoxia, and energy crisis resulting in accelerated purine degradation) upregulate this enzyme's oxidoreductase form, accelerating the transformation of hypoxanthine to xanthine and ultimately UA (Fig. 1). The activity of XO increases in pathological conditions (Kuppusamy et al. 2005), which is attributed to the increased release of XO from the liver into the vascular system. The excessive release of XO occurs in conjunction with activation of the pentose phosphate pathway, which promotes the activation of the hepatic de novo purine synthetic pathway, thereby triggering XO activity. This increased XO activity is involved in adipose tissue differentiation by regulating the expression of PPAR-γ at early stages of cell differentiation (Cheung et al.). It explains the positive association of XO activity and waist circumference (WC), triglycerides (TG), markers of inflammation, the level of homeostatic model assessment of insulin resistance (HOMA-IR), and body mass index (BMI). Furthermore, obesity also induces tissue hypoxia by activating the hypoxia-inducible factor (HIF). The chronic activation of HIF causes IR and hypoxia. The XO activity is also controlled by HIF expression, and overactivation of HIF can lead to higher XO activity (Majmundar et al.). Therefore, one can easily establish that measuring the XO activity in undiagnosed hyperglycemic and/or overweight/obese individuals can be an early signature of disrupted glycolysis, obesity-induced diabetes, and diabetic complications. It is also pertinent to note that the above-mentioned

pathophysiological conditions are common in prediabetic and/or obese subjects susceptible to diabetes. Numerous studies evidenced the positive relationship of XO with T2DM occurrence in a susceptible population (Table 1). A direct relationship of XO activity with BMI and the role of XO in adiposity and inflammation have been reported in 2017 (Washio et al. 2017). Later, four population-based cohort studies found a positive association of XO activity with BMI, WC, systolic blood pressure (SBP), diastolic blood pressure (DBP), hepatic enzymes (aspartate transaminase, AST; alanine transaminase, ALT; gamma-glutamyl transpeptidase, GTP), UA, TG, estimated glomerular filtration rate (eGFR), fasting blood glucose (FBG), IR, HOMA-IR, glycated hemoglobin (HbA1c), and xanthine in lean diabetic, dyslipidemic, and hypouricemic patients (Furuhashi et al. 2019). Recently, Sunagawa et al. (2019) reported the highest XO activity in diabetic patients followed by metabolic syndrome and healthy lean subjects. This study also presented a positive correlation of XO activity with indices of IR and the level of circulating hepatic transaminases. Similar results have been published in a more recent study (Okuyama et al. 2021). Elevated XO activity was found correlated with increased hepatic glucose production and fasting insulin secretion (Varadaiah et al. 2019). In another study performed on obese and lean prepuberty children, an increased XO activity (by 16%), leptin (tenfold), and interleukin 6 (IL-6) to C-reactive protein ratio (threefold) in obese versus lean children were observed (Chiney et al.). In another cross-sectional study, XO activity was measured, and the highest literature-find increase of XO activity (3.8-fold) was reported (Tam et al. 2014). The increased XO activity is also in line with the increase of such glycemic parameters as HbA1c, FBG, and oxidative stress markers like malondialdehyde (MDA) and superoxide (Kuppusamy et al. 2005). XO activity was also reported as an independent risk factor of metabolic syndrome and cardiovascular disease (CVD) in a general Japanese population (Kotozaki et al. 2021). Additionally, the relationship of IR with XO activity was also revealed (Kurajoh et al. 2019).

Upregulated XO accelerates the formation of MDA and superoxides, therefore elevated serum level of XO is also a biomarker of oxidative stress. It has been reported that overexpression of XO (>200%) increased the ratio of oxidized to reduced glutathione GSSG/GSH by 300% and MDA level by 50% in diabetic subjects compared to the healthy control group (Desco et al.).

The studies mentioned above indicate that XO activity can be used as a clinically relevant biomarker to monitor the development of metabolic syndrome and T2DM in susceptible undiagnosed populations and follow the progress of diabetes and diabetes-related complications.

Adenosine Metabolizing Enzymes, Inosine and Adenosine

Adenosine is produced from AMP by the action of 5′-nucleotidase (5′-NT) and is converted back into AMP by adenosine kinase (Adok) or into inosine by adenosine deaminase (ADA) (Vannoni et al. 2004). ADA is a polymorphic enzyme involved in the irreversible transformation of adenosine to inosine. An increase of ADA is

Table 1 Activities or concentrations of xanthine oxidase reported in referred studies

Reference	Type of the study	Participants	Age of participants (years)	Studied correlation	XO concentration or activity	
					Controls	Patients
(Kuppusamy et al. 2005)	RCT, cross-sectional study	Diabetic and aged-matched healthy controls $n = 650$ and $n = 280$	35–80	XO with FBG, HbA1C, SOD, Cat, GPx, FRAP, MDA, glycemic control index, Hs-CRP, UA	0.30 ± 0.14 U/l	0.87 ± 0.15 U/l
(Washio et al.)	Preliminary study	General participants, $n = 29$	25–28	XO vs BMI, hs-CRP, IR	–	3.4 ± 0.8 (pmol/h/mL)
(Furuhashi et al.)	Population-based cohort study	General participants, $n = 627$ (male/female: 292/335)	65	XO with BMI, TG, TC, HDL-C, FBG, HbA1C, glycemic control index, Hs-CRP, UA	32.3 pmol/h/mL of plasma	53.4 pmol/h/mL of plasma
(Sunagawa et al. 2019)	Cross-sectional analytical study	Lean patients without diabetes and patients with T2DM, $n = 5$, $n = 51$	55–70	XO vs indices of IR and hepatic transaminases	0.13 pmol of IXP/min/mL	0.55 pmol of IXP/min/mL
(Okuyama et al. 2021)	Cross-sectional analytical study	Japanese patients with T2D, $n = 94$	64–85	XO vs indices of IR and hepatic transaminases	–	67.7 (31.1–184) (pmol/h/mL)
(Chiney et al.)	RCT, cross-sectional study	Obese and aged-matched healthy controls $n = 9$ and $n = 16$	6–10	XO with NAT2, CYP1A2	0.6 ± 0.047 mmol/L	0.7 ± 0.06 mmol/L
(Tam et al. 2014)	RCT, cross-sectional study	Obese and aged-matched healthy controls $n = 20$ and $n = 22$	12–13	XO with BMI, WC, HDL-C, adiponectin, MCP-I, LDL-C	31 ± 2.14 units/g of protein	121 ± 7.1 units/g of protein

(continued)

Table 1 (continued)

Reference	Type of the study	Participants	Age of participants (years)	Studied correlation	XO concentration or activity	
					Controls	Patients
(Kotozaki et al. 2021)	Population-based cohort study	General participants, $n = 1737$ (males/females = 577/1160)	64–66	XO with BMI, UA, dyslipidemia, diabetes, CVD	34.8 (20.8–62.7) (pmol/h/mL of plasma)	43.7 (25.3–77.3) (pmol/h/mL of plasma)
(Kurajoh et al. 2019)	Cross-sectional study	General participants, $n = 193$ (92 males and 101 females)	47–67	XO vs HOMA-IR, BMI, age, sex, adiponectine, HbA1c	1.50 ± 0.44 (pmol/h/mL)	76.8 ± 45.8 (pmol/h/mL)

Controls are referred to as participants without diabetes, obesity, and metabolic syndrome with normal glucose values and insulin indices. Patients are referred to as participants with diabetes or obesity with abnormal glucose and/or insulin indices. Body mass index (BMI); cardiovascular disease (CVD); catalase (Cat); cytochrome P-4501A2 (*CYP1A2*); fasting blood glucose (FBG); ferric reducing antioxidant power assay (FRAP); glutathione peroxidase (GPx); glycated hemoglobin (Hb1Ac); high-density lipoprotein *cholesterol* (HDL-C); high-sensitivity C-reactive protein (hs-CRP); homeostatic model assessment for insulin resistance (HOMA-IR); insulin resistance (IR); low-density lipoprotein *cholesterol* (LDL-C); *malondialdehyde* (*MDA*); monocyte chemoattractant protein-1 (*MCP*-1); N-acetyltransferase 2 (*NAT2*); randomized controlled trial (RCT); superoxide dismutase (*SOD*); triglycerides (TG); total cholesterol (TC); type 2 diabetes mellitus (T2DM); uric acid (UA); waist circumference (WC); Xanthine oxidase (XO)

observed in inflammatory diseases and T2DM or hyperglycemia, conditions associated with low-grade systemic inflammation (Bopp et al. 2009). Adok is a high affinity and low capacity enzyme that plays an integral role in energy homeostasis by regulating the metabolic clearance of adenosine. Generally, Adok is found in all forms of life and is involved in several biological functions like the stimulation of glucose transport and oxidation, lipolysis, modulation of blood flow and endogenous response to ischemia, inflammation, immune response, neurotransmission, and pain, including clearance of adenosine by phosphorylation of adenosine to form AMP (Xu et al. 2017). The intercellularly produced adenosine is transported to extracellular space mainly via specific bidirectional transporters (Oyarzún et al. 2015). Adok activity is under the effect of various modulators such as intracellular pH, insulin, nitric oxide (NO), glucose, inorganic phosphate, and inflammatory cytokines (Fredholm et al. 2007; Park et al. 2006). The expression level of both enzymes is contradictory to each other. It is primarily believed that the expression of ADA and Adok is compensatory to regulate the adenosine level. The ADA-led transformation of adenosine to inosine is a rate-limiting step and controls the serum concentration of adenosine and inosine. The concentrations of both metabolites are well-regulated unless pathophysiological conditions increase the ADA expression concurrent with XO expression. High expression of ADA accelerates adenosine-to-inosine bioconversion, depleting the adenosine and increasing the inosine level. The depleting adenosine level is detrimental to metabolic health and cause the IR. The significance of adenosine can be judged from its multipurpose role in glucose metabolism and insulin secretion. Adenosine stimulates the insulin activity by activating numerous pathways including lipid synthesis, glucose transport, leucine oxidation, and pyruvate dehydrogenase activity. It potentiates the action of insulin in liver, increases insulin sensitivity for glucose transports, and accessibility of glucose transporter type 4 (GLUT-4) to cell surface for glucose transportation, as well as gluconeogenesis and glycogenolysis via increasing cyclic AMP (cAMP). Adenosine also mimics the action of insulin on lipid and glucose metabolism and showed antilipolytic properties in adipose tissue (Johansson et al. 2008).

Serum level of ADA was found increased and in direct relationship with insulin, HOMA-IR, total cholesterol (TC), and age in women with metabolic syndrome (De Bona et al. 2013). Similar results were obtained in a case-control study conducted on Latin American patients with metabolic syndrome. Substantial increase of HbA1c, HOMA-IR, TC, low-density lipoprotein cholesterol (LDL-C), proinflammatory cytokines, and chemokines were observed, and a close relationship of these parameters with an elevated level of ADA was reported (Chielle et al. 2018). Moreover, it is worthy to note that elevated ADA level was in a reciprocal relationship with insulin receptor 1 (IRS-1) and GLUT-4 in the liver and muscle tissues of the patients with metabolic impairment. The reduction in the expression of IRS-1 and GLUT-4 (in combination with elevated ADA) prompted the production of IL-6, high-sensitivity C-reactive protein (hs-CRP), and free fatty acids (FFA), which interfere with the normal glucose metabolism by inducing the reduction of adiponectin (Chielle et al. 2018). The altered ADA activity was also used as a marker discriminating nonobese from obese and nondiabetic from diabetic patients.

The ADA and isozyme activities were higher in obese and diabetic individuals than in lean and diabetic individuals. Likewise, ADA was found overexpressed in diabetic people in comparison to healthy lean controls. This study also showed that ADA activity is a hallmark of inflammation in obese-diabetic patients (Sapkota et al. 2017). In another study a positive association of increased ADA level with glycemic parameters was reported (Khemka et al. 2013). ADA is also considered the main regulator of insulin bioactivity and was reported as a marker of insulin function (Larijani et al. 2016). In another two case-control studies, the positive correlation of ADA with lipid profile (except high-density lipoprotein cholesterol, HDL-C), HbA1c, hypertension, smoking, and FBG in Indian (Malathi 2017) and Chinese (Xuan et al. 2019) diabetic patients was observed.

Quantification of purines and pyrimidines was performed in two case-control studies revealing elevated levels of inosine and other nucleic acid-based metabolites in patients with diabetes and diabetic nephropathy (DN). Compared to healthy controls, higher concentrations of xanthine, UA, adenosine, and inosine in DN patients were observed. Adenosine and inosine lost their statistical significance in T2DM vs healthy subjects comparison. These results were challenged by a multi-omics study aiming to find early diagnostic markers of T2DM and DN. This study proposed an integrated biomarker system composed of 40 metabolites, 5 genes, and 6 clinical parameters to differentiate healthy subjects from T2DM and DN patients, as well as for DN staging. In this study, inosine was found the second most reliable biomarker with total predictive accuracy up to 97% and the highest specificity and sensitivity. Interestingly, the cutoff value of inosine was used in combination with the glomerulus filtration rate to differentiate DN stages (Huang et al. 2013).

Metabolomics studies also discovered that purine metabolites were significant in relation to diabetes and related diseases. In an NMR-based metabolomics study, inosine was found as one of the most robust and reliable biomarkers of nonalcoholic steatohepatitis, a condition connected to a future risk of T2DM (Kimberly et al. 2017). In another study, 11 metabolites were found strongly associated with BMI and incidence of T2DM, and three of them were purine degradation products (Ottosson et al. 2019). Investigations on obese yet nondiabetic patients delineated a strong association of inosine, xanthine, and hypoxanthine with BMI, fasting glucose level, and HOMA-IR. An increase of purinergic metabolites, including inosine, was observed in diabetic individuals in comparison to healthy controls. Still, inosine did not vary significantly when comparison was made between diabetic patients with and without DN (Hirayama et al. 2012). In a similar study, increased levels of inosine and adenosine were noted in diabetic people compared to non-diabetic subjects. Although both metabolites' levels were higher while comparing diabetic subjects with and without DN, statistical significance was not achieved. This work bespeaks the significance of inosine and adenosine in monitoring the progress of T2DM. The same research group also published another metabolomics work focusing on diabetes and diabetic retinopathy (DR). This study noticed higher levels of inosine and adenosine in DR patients than in diabetic and healthy participants. However, the variation in the level of inosine and adenosine was statistically insignificant when comparing diabetic and healthy controls. This work proposed

inosine as an excellent biomarker for monitoring the progress of DR with 68.2% sensitivity and 81.3% specificity. Besides DR, DN, and kidney problems, metabolic profile based on purine metabolites was also used to distinguish healthy, diabetic-periodontitis, and nondiabetic-periodontitis subjects. The discriminatory metabolites were increased in nondiabetic periodontitis patients compared to healthy subjects (Barnes et al. 2014). An opposite trend was observed when an evaluation was made between diabetic people with periodontitis and healthy controls, suggesting an interaction between the periodontal signature and diabetes signature. There has also been seen a decreased redox balance capacity and increased purine degradation in diabetic-gingivitis/diabetic-periodontitis patients resulting in increased plasma/saliva concentration of inosine (1.7-fold) and adenosine (1.37-fold) (Barnes et al. 2014). The association of raised ADA in diabetic patients with other diseases in which systematic inflammation is observed (experimental colitis, coronary artery calcification, acute lymphoblastic leukemia, autoimmune hepatitis, and rheumatic disease) was also reported (Yu et al. 2021). Recent studies have also illustrated that the predictive value of xanthine and inosine for T2DM risk applies primarily to individuals with a specific genetic variant of the TCF7L2 gene (rs7903146 T-allele). There is also evidence that the genetic variant of the TCF7L2 gene and single-nucleotide polymorphism (SNP) interact with the purine catabolism. It was also found that higher levels of plasma xanthine and inosine are connected with a progressive T2DM risk in individuals with TT alleles on rs7903146 than the combined CC genotype (Papandreou et al. 2019). Largely, these findings stress the significance of sketching variations across the whole purine pathway, not just its end products, and interactions with other regulation levels.

Human studies providing quantitative values for the above-mentioned metabolites in control and diabetic patients are summarized in Table 2.

Inosine-5′-Monophosphate Dehydrogenase

Inosine-5′-monophosphate dehydrogenase (IMPDH) is an oxidoreductase enzyme that catalyzes the rate-limiting step in the de novo biosynthesis of guanine nucleotides, i.e., NAD^+-dependent oxidation of inosine monophosphate (IMP) to xanthosine 5′-monophosphate (XMP). Human IMPDH has two isoforms, *h*IMPDH1 and *h*IMPDH2, having the same amino acid sequence (514 amino acids) in 85% but different concerning the inhibition rate and affinity (Cuny et al. 2017). Every subunit of isozyme was found to have two crucial domains, the (β/α)8 barrel catalytic domain and cystathionine beta-synthase (CBS) domain. The catalytic cysteine-binding site (which binds IMP) is highly conserved, whereas the NAD^+ binding site is more variable. The conversion of IMP to XMP is completed in a two-step process: the first step involves the addition of IMPDH's Cys331 to IMP followed by hydride transfer to NAD^+ to generate an IMPDH-IMP conjugate and NADH. The second step is the hydrolysis of IMPDH-IMP to generate XMP and IMPDH (Cuny et al. 2017). The plasma/serum levels of XMP/IMPDH were found altered in diabetic subjects compared to healthy subjects. It was proposed that XMP/IMPDH

Table 2 Concentrations of adenosine deaminase reported in referred studies

Reference	Type of the study	Participants	Age of participants (years)	Studied correlation	Concentration	
					Controls	Patients
(De Bona et al. 2013)	Case-control study	Healthy subjects and MetS patients, $n = 48$, $n = 39$	42–55	ADA	17.26 ± 7.51 U/L	17.18 ± 7.97 U/L
(Chielle et al. 2018)	Case-control study	MetS and healthy controls, $n = 48$, $n = 36$	30–46	ADA with glycemic index and proinflammatory cytokines	5.9 ± 1.1 U/L	7.7 ± 1.5 U/L
(Sapkota et al.)	Cross-sectional analytical study	Healthy control and T2DM, $n = 80$ and $n = 80$	35–70	ADA to glycemic indices	8.0 ± 2.24 U/L (ADA1); 12.29 ± 2.23 U/L (ADA2)	14.19 ± 4.U/L (ADA1); 21.9 ± 4.42 U/L (ADA2)
(Khemka et al. 2013)	Case-control study	Lean diabetic and healthy controls, $n = 56$ and $n = 45$	52–55	ADA to glycemic indices	17.02 ± 5.74 U/L	38.77 ± 14.29 U/L
(Larijani et al. 2016)	Case-control study	Diabetics and healthy controls, $n = 33$, $n = 35$	60–65	ADA1 and ADA2 to glycemic indices	6.0 ± 6.24 U/L (ADA1); 7.66 ± 1.33 U/L (ADA2)	13.0 ± 5.19 U/L (ADA1); 8.5 ± 3.96 U/L (ADA2)
(Malathi 2017)	Case-control study	Diabetics and healthy controls, $n = 54$, $n = 55$	30–70	ADA to glycemic indices	17.96 ± 6.83 U/L	33.93 ± 11.74 U/L
(Xuan et al. 2019)	Case-control study	Diabetics and healthy controls, $n = 5212$, $n = 4717$	60–75	ADA to glycemic indices, hypertension, smoking, age, BMI,	11.71 ± 4.20 U/l	10.08 ± 3.57 U/l

Controls are referred to as participants without diabetes, obesity, and metabolic syndrome with normal glucose values and insulin indices. Patients are referred to as diabetes or metabolic syndrome participants with abnormal glucose and/or insulin indices. Adenosine deaminase (ADA); body mass index (BMI); metabolic syndrome (MetS); type 2 diabetes mellitus (T2DM)

can be considered a reliable, robust and sensitive early biomarker of diabetes (both T1DM and T2DM) (Sobiak et al. 2020). Many studies monitored the plasma/serum level of IMPDH in various cohorts and concluded significant intergroup and intraindividual variation in plasma/serum levels of XMP/IMPDH (Budde et al. 2007a, b). However, there is a lack of prospective/longitudinal studies in the literature, which could document the level of IMPDH in the samples with event of diabetes occurrence through the study duration. Many studies reported significantly plummeted plasma/serum level of IMPDH after the incidence of diabetes (17.5 ± 2.8 nmol of XMP/h/μg of protein) (especially in kidney transplant patients) as compared to counterpart healthy population (46.6 ± 2.5 nmol of XMP/h/μg of protein in nondiabetics) (Dostalek et al. 2013).

Uric Acid

Overproduction and/or underexcretion of UA is generally known as hyperuricemia (Cicero et al. 2021). It can be related to genetic predisposition (Bleyer and Hart 2006), but serum uric acid (SUA) level can also be regulated by a diet (Skoczyńska et al. 2020) or physical activity (Hou et al. 2021). Hyperuricemia is diagnosed when blood UA level exceeds 7 mg/dL for men and 6 mg/dL for women (Hou et al. 2021). There are three main reported reasons for hyperuricemia, i.e., overproduction of UA through the perturbed purine degradation pathway, renal underexcretion of UA, and gut underexcretion of UA. Underexcretion usually occurs through the disturbance in the two reuptake transporters, i.e., urate transporter 1 (URAT-1) and glucose transporter 9 (GLUT-9), as well as one secretory transporter (ATP-binding cassette subfamily G-ABCG2), whereas perturbed purine degradation is considered a metabolic disorder (Ichida et al. 2012).

The relationship between hyperuricemia with hyperglycemia is well documented in the literature. The hyperglycemic condition causes the higher flux of glucose-6-phosphate through the shunt pathway due to impairment of normal glycolytic pathways, resulting in increased UA production. The overactivation of this shunt-pathway is also a primary cause for enhanced lipogenesis in hyperinsulinemia (Gill et al. 2013). Hyperuricemia has also been closely associated with obesity and metabolic disorders (i.e., diabetes, dyslipidemia, hyperglycemia, and insulin resistance-IR). The relationship between SUA level and obesity, T2DM, and hyperuricemia was investigated in many cohort studies of different ethnicities. The Doetinchem Cohort Study concluded a positive association of higher SUA level, high sensitivity creative protein, and γ-glutamyltransferase (GGT) with increasing BMI and age (Hulsegge et al. 2016). The relationship between elevated SUA level and fat mass, SBP, HOMA-IR and HbA1c, TG, and TG/ HDL-C ratio was recently demonstrated (Foster et al. 2020). Another study noted a strong positive correlation of increased SUA level with TG level in black males. The authors also identified a correlation of SUA level with age, BMI, alcohol use, and hypertension, while SUA was not correlating with IR, HbA1c, and fasting plasma glucose (Conen et al. 2004). Likewise, elevated SUA also showed a similar relationship with metabolic syndrome

parameters of overweight but healthy subjects. The authors further studied the linkage of SUA and inflammatory biomarkers by stratifying the population based on the SUA level. The higher quartiles population showed progressively higher proinflammatory markers (hs-CRP, fibrinogen, erythrocyte sedimentation rate, ferritin, and complement C3). In addition, increasing SUA was also found to activate the IκB Kinase–NF-κB pathway by promoting the dose-dependent phosphorylation of IKK at Ser-180 site and IκBα at Ser-32 site (up to 2.0-fold over basal) (Spiga et al. 2017). A positive correlation between the elevated plasma levels of TG and FFA (independently of fat distribution) and hyperuricemia was also observed (Hayden and Tyagi 2004). In the study investigating the relationship of BMI, leptin, and IR, the authors suggested leptin as the regulator of serum UA, obesity, and hence IR (Bedir et al. 2003). Another longitudinal study performed on Japanese youth reported an elevated level of SUA in obese and hypertensive subjects. This work further elaborated that SUA level at the entry point can be used as a reliable determinant factor predicting changes in BMI, blood pressure, and IR (Masuo et al.). The role and correlation of elevated SUA with the risk of T2DM was further evaluated and affirmed by a longitudinal prospective study on healthy Japanese males but with different BMI. The study manifested higher rates of IR in individuals with BMI > 24.2 Kg/m^2 for any level of SUA (Nakanishi et al.). In another study, SUA concentration was measured in hemodialysis patients over a period of 6 months. The authors reported the highest rate of T2DM incidence in lean males with increasing SUA level even after adjustment for such confounding factors as hypertension and IR. People in the upper quintiles showed 58–78% more vulnerability toward T2DM incidence than those in lower quartiles. Obtained results also suggested that increased SUA may also induce lipogenesis in hepatocyte and mitochondrial oxidative stress, especially in undiagnosed prediabetic people (Lanaspa et al. 2012). A recent cohort study performed on prediabetic patients showed a positive correlation of baseline SUA level with WC and fasting plasma glucose. This study found that in prediabetic patients, every SUA increase of 1 mg/dl was associated with the increase of FPG by 0.082 mg/dl (Anothaisintawee et al. 2017).

Recently, one nested, cross-sectional case-cohort study identified the association of peripheral purine metabolites and UA levels as risk factors for T2DM. The increased level of UA was found positively associated with the risk of T2DM at baseline. Spearman's correlation revealed a positive relationship between baseline HOMA-IR and fasting levels of UA, guanosine, xanthine, and adenosine, as well as xanthine/hypoxanthine ratio. The inverse association was seen in the case of allantoin/uric acid and inosine/adenosine ratios (Papandreou et al. 2019). The ability of the SUA level to predict T2DM was investigated in age-stratified individuals of Italian origin. This work detected nonsignificant relation of baseline SUA with an incidence of T2DM after adjustment to sex, blood pressure, HbA1C, serum TG, smoking habits, HDL-C, use of diuretic drugs, WC, and alcohol consumption. However, while focusing on the patients with median age > 46.8 years, an increase in standard deviation (SD) of 1 SUA considerably increased the risk of T2DM (by 29%) and appearance of impaired fasting glucose (2.5-fold) (Bombelli et al.

2018). Another cohort study on the Mediterranean population also indicated the elevated SUA as a risk factor for T2DM in hypertensive patients (Viazzi et al. 2011). Furthermore, univariate analysis revealed a positive correlation of SUA level with FBG, BMI, HOMA-IR, waist-hip ratio, blood pressure (BP), and serum insulin concentration. It has been observed that an increase in the concentration of uric acid by 3 mg/dL from the baseline can cause 87% more chances of mild hypertension and early insulin resistance (Yoo et al.).

As stated above, SUA level was found to be linearly increased with fasting insulin level and HOMA-IR. One can easily regard UA as a surrogate biomarker of metabolic disorders. Subsequently, many studies also used this biomarker in conjunction with xanthine to stage the progress of T2DM and DN (Chen et al. 2018). Higher plasma levels of UA and xanthine were detected in patients with advanced stages of T2DM and DN. In addition to this, SUA level is also considered a risk factor of DN in diabetic patients, and many studies also correlated its level directly with the initial IR (Chen et al. 2018).

In summary, the presented studies found relevancy between elevated SUA at the baseline and onset of future obesity and/or diabetes and the association of elevated SUA and various parameters of diabetes and diabetic complications. The level of SUA was also found associated with hypertension, hyperlipidemia, hypertriglyceridemia, and IR. Human studies providing SUA quantitative values in control and diabetic patients are summarized in Table 3.

Conclusion

The development of insulin resistance or otherwise impaired metabolic conditions affects a number of metabolic pathways, including purine metabolism. The data suggest that any undiagnosed metabolic impairment directly influences purine metabolism by altering the expression of their key enzymes and their metabolites, which worsens the sensitivity and release of insulin and influences the hexoses transportation and metabolic manipulation. The findings showed that all the prominent and rate-limiting enzymes and metabolites related to purine metabolism were altered in participants that eventually developed diabetes when compared with a control group. XO, ADA, adenosine, inosine, and others were found elevated in the biofluids of subjects with IR or T2DM. Catabolism of purine nucleosides ultimately leads to the production of UA, which was also found elevated in the subjects with metabolic syndrome in comparison to controls.

Applications to Prognosis, Other Diseases or Conditions

In this chapter changes in the level of key purine enzymes and related metabolites in relation to type 2 diabetes, prediabetes, and obesity is discussed. The levels of xanthine oxidase, adenosine deaminase, inosine, adenosine, Inosine-5′-monophosphate dehydrogenase, as well as uric acid, and their correlations with insulin resistance,

Table 3 Concentrations of serum uric acid reported in referred studies

Reference	Type of the study	Participants	Age of participants (years)	Studied correlation	Concentration	
					Controls	Patients
(Xia et al.)	Cross-sectional study	Healthy controls ($n = 31$), diabetics ($n = 23$), DN ($n = 65$)	55.0 ± 7.5	SUA vs TG, HDL-C, Leptin, BP, glucose, HbA1C and serum insulin	45 ± 2.3 mg/L	65 ± 1.4 mg/L
(Xia et al.)	Cross-sectional study	Healthy controls ($n = 40$), T2DM ($n = 35$), T2DM with DR ($n = 39$)	54.4 ± 5.4 (Controls) 55.9 ± 7.0 (T2DM) and 56.5 ± 5.4 (T2DM with DR)	SUA vs T2DM and T2DM with DR	46.63 ± 2.41 mg/L	T2DM: 53.81 ± 2.36 mg/L T2DM with DR: 70.55 ± 3.97 mg/L
(Gill et al. 2013)	Cross-sectional study	Healthy ($n = 50$), diabetics ($n = 50$)	40–65	SUA vs HbA1C and serum insulin	2 ± 1.19 mg/dL	6.6 ± 0.5 mg/dL
(Hulsegge et al. 2016)	Longitudinal, prospective	Four generation of normal population ($n = 5{,}155$) followed for 15 years	From 26 to 65 at baseline	SUA level at baseline was 0.24 ± 0.06 mmol/L for women and 0.33 ± 0.06 mmol/L for men. It increased with age, particularly in participants whose BMI increased		
(Foster et al. 2020)	Retrospective study	General participants, $n = 100$	13.9 ± 2.0	SUA vs fat mass, SBP, HOMA-IR, HbA1c, and TG	295.2 ± 47.9 mmol/L	414.1 ± 51.8 mmol/L (hyperuricemia)
(Spiga et al. 2017)	Cross-sectional study	Overweight but healthy adult $n = 2731$	47.6 ± 13.5	A positive relationship between SUA (average 5.0 ± 0.034 mg/dL) and acute-phase reactants, such as high-sensitivity CRP, fibrinogen, ferritin, complement C3, and erythrocyte sedimentation rate		
(Bedir et al.)	Cross-sectional study	General population, $n = 420$ (210 men and 210 women)	48.7 ± 1.28	SUA vs TG, HDL-C, Leptin, glucose, HbA1C and serum insulin	248.0 ± 4.76 mmol/L	311.6 ± 5.35 mmol/L

(Masuo et al.)	Longitudinal study	Young, non-obese, normotensive men, $n = 433$	< 50	BMI, BP, and levels of SUA, fasting plasma norepinephrine, insulin, and leptin	4.1 mg/dL	>5.6 mg/dL
(Lanaspa et al. 2012)	Cross-sectional study	Chronic hemodialysis patients, $n = 546$	56.3 ± 16.2	SUA in relation to hepatic steatosis and mitochondrial oxidative stress	From 4.9 ± 0.9 to 7.4 ± 1.1 mg/dL	From 5.2 ± 0.8 to 7.8 ± 0.9 mg/dL
(Anothaisintawee et al. 2017)	Cross-sectional study	Prediabetes patients, $n = 1633$	62.76 ± 8.9	SUA vs WC and FBG	Control group not included	5.79 ± 1.46 mg/dL
(Bombelli et al.)	Prospective, observational study	General population, $n = 1677$	25 to 74	SUA as a potential risk factor for developing diabetes and cardiovascular disease	3.6 mg/dL	>4.8 mg/dL
(Viazzi et al.)	Cross-sectional study	Caucasian hypertensive patients, $n = 758$	49 ± 10	SUA levels ($\geq$318 μmol/l for women and $\geq$ 420 μmol/l for men) at baseline were associated with a significantly higher risk of developing diabetes		
(Yoo et al.)	Cross-sectional study	General population, $n = 53{,}477$ (34,169 males, 19,308 females)	40.7 ± 9.1	SUA vs hypertension, insulin resistance, and the risk factors of metabolic syndrome	5.3 ± 1.4 mg/dL (from <4.2 to >6.29 mg/dL)	

Controls are referred to as participants without diabetes, obesity, and metabolic syndrome with normal glucose values and insulin indices. Patients are referred to as participants with diabetes, prediabetes, or obesity with abnormal glucose and/or insulin indices. Blood pressure (BP); body mass index (BMI); diabetic nephropathy (DN); diabetic retinopathy (DR); fasting blood glucose (FBG); glycated hemoglobin (Hb1Ac); high-density lipoprotein cholesterol (HDL-C); serum uric acid (SUA); systolic blood pressure (SBP); total cholesterol (TC); triglycerides (TG); type 2 diabetes mellitus (T2DM); waist circumference (WC)

prediabetes, and diabetes have been reviewed. Reviewed studies showed that all the prominent and rate-limiting enzymes and metabolites related to purine metabolism were altered in participants with diabetes when compared to a control group. Moreover, the abovementioned components of purine metabolic pathways were found correlated with BMI, hepatic enzymes, cholesterol, or blood pressure. Xanthine oxidase, adenosine deaminase, adenosine, inosine, and others were found elevated in the biofluids of subjects with IR or T2DM. Except relation to diabetes, xanthine oxidase was found related to cardiovascular disease and dyslipidemia, while adenosine deaminase to hypertension. Serum uric acid levels were found additionally related to hypertension, cardiovascular disease, and metabolic syndrome. Some of the evaluated parameters (e.g., isopentenyladenosine-5-monophosphate, uric acid, xanthine, and xanthine oxidase) were reported as type 2 diabetes predictive biomarkers. Selected components of the purine metabolism pathways can be used as biomarkers for the early diagnosis of diabetes or prediabetes and evaluation of the risk of diabetes development.

Mini-Dictionary of Terms

T2DM: Most commonly diagnosed type of diabetes, characterized by abnormal glucose and lipid metabolism, resulting from resistance to the effects of insulin and insufficient response to the secretion of this hormone. As this disease progresses, a partial β-cell insufficiency and deficiency in insulin production may also occur.

Purinosome: A dynamic multi-protein complex system involved in the de novo biosynthesis of purines.

Xanthine oxidase: An enzyme found in many species, including humans. It is required to produce uric acid by the breakdown of purine nucleotides. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. It generates reactive oxygen species.

Adenosine deaminase: This is a metalloenzyme found in microorganisms, plants, invertebrates, and all mammalian cells that is involved in purine metabolism. It catalyzes the irreversible deamination of adenosine to inosine and deoxyadenosine to deoxyinosine.

Biomarker: A measurable biological feature that can distinguish the normal condition from pathological condition or indicate a response to an administered therapeutic drug.

Key Facts of Purine Enzymes and Related Metabolites

- Under normal physiological conditions, purine levels in cells are maintained by the coordinated action of the de novo biosynthesis and salvage pathways.

- Purine de novo biosynthesis is an energy-intensive ten-step process involving six enzymes that rely on the substrates from amino acids and carbohydrates metabolism from the TCA cycle.
- For each molecule of IMP generated, five molecules of ATP, two molecules of glutamine and formate, and one molecule of glycine, aspartate, and carbon dioxide are needed.
- Concentrations of purine enzymes and their metabolites are altered in individuals with obesity, prediabetes, and metabolic syndrome.
- The components of the purine metabolism pathways can be considered early biomarkers for the screening, early diagnosis, and monitoring of diabetes and related complications.

Summary Points

- The development of insulin resistance or other metabolic impairment affects purine metabolism, altering its key enzymes and metabolites.
- The activity of xanthine oxidase can be used as a clinically relevant biomarker to monitor the development of the metabolic syndrome and type 2 diabetes in susceptible undiagnosed populations and follow the progress of diabetes and diabetes-related complications.
- Adenosine deaminase, adenosine, and inosine were found elevated in the biofluids of subjects with insulin resistance and type 2 diabetes.
- Catabolism of purine nucleosides leads ultimately to the production of uric acid.
- Elevated serum uric acid is a risk factor for future obesity and/or diabetes development.
- The level of serum uric acid was found associated with hypertension, hyperlipidemia, hypertriglyceridemia, and insulin resistance.

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Serpin A12 (Vaspin) as a Serine Protease Inhibitor

6

From Functional Mechanisms to Applications as a Biomarker in Diabetes

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Contents

Introduction 155
Type 2 Diabetes and the Importance of Biomarkers 156
Vaspin as a Diabetes Biomarker 157
Biochemical Characterization 157
Main Sources of Vaspin 157
Vaspin and Anti-Inflammatory Effects 158
Glucose Intolerance and Vaspin 159
Insulin Role and Vaspin Action 161
Circulating Vaspin Level, Type 2 Diabetes, and Obesity 162
Metabolic Syndrome (MetS) and Vaspin 162
Applications to Prognosis, Other Diseases, and Conditions 163
Conclusion 164
Mini-Dictionary of Terms 164
Key Facts of Type 2 Diabetes 165
Summary Points 165
References 165

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Abstract

Wide-ranging studies have recently been performed on adipokines and their association with metabolic processes. Vaspin, a serpin-derived adipokine, is an essential linker between metabolic alterations, obesity, and insulin resistance. Vaspin mRNA is expressed in different tissues and has different roles in various tissues. This adipokine has anti-inflammatory and anti-apoptotic effects and is considered an insulin sensitizer whose intracellular and extracellular levels are affected by metabolic changes. Because vaspin was first identified in the visceral adipose tissue of a rat model of type 2 diabetes and obesity, various laboratory and clinical studies have been performed to introduce it as a biomarker of diabetes. The incidence of insulin resistance in type 2 diabetes is associated with the function of vaspin as an insulin sensitizer, which is quite different from the mechanism of type 1 diabetes. This chapter discusses the studies conducted in recent years about the probability of recognizing vaspin as a biomarker for the prognosis and diagnosis of type 2 diabetes. The potential role of vaspin in the body, molecular mechanisms, and signaling pathways associated with vaspin and type 2 diabetes also has been reviewed to achieve the aim of the chapter. It is concluded that circulating vaspin level may be considered as a potential biomarker in assessing type 2 diabetes and the prognosis of its complications.

Keywords

Biomarker · Insulin resistance · Type 2 diabetes · Serpin A12 · Vaspin

Abbreviations

ABCC8	ATP-binding cassette subfamily C member 8
ADMA	Asymmetric dimethylarginine
AMI	Acute myocardial infarction
ASP	Acylation-stimulating protein
C-peptide	Connecting peptide
CrP	C-reactive protein
DPP-4	Dipeptidyl peptidase 4
FGF21	Fibroblast growth factor 21
FGT	Fasting glucose test
GRP78	Glucose-regulated protein
HAEC	Human aortic endothelial cell
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostatic model assessment for insulin resistance
IGF-1	Insulin-like growth factor-1
IL-1b, IL-6, IL-10	Interleukin
IRS	Insulin receptor substrate
KLK7	Kallikrein-related peptidase 7
MetS	Metabolic syndrome
NGT	Normal glucose tolerance

NO	Nitric oxide
NOX	NADPH oxidase
OLETF	Otsuka Long-Evans Tokushima fatty
PDGF-BB	Platelet-derived growth factor-BB
PDGFR	PDGF receptor
PI3K/AKT, MAP kinase	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
RBP4	Retinol-binding protein 4
RCL	Reactive center loop
ROS	Reactive oxygen species
RYGB	Roux-en-Y gastric bypass
SAA3	Serum amyloid A3
SNPs	Single nucleotide polymorphisms
T2DM	Type 2 diabetes mellitus
TGFβ	Transforming growth factor-β
TNFα	Tumor necrosis factor-α
Vaspin	Serpin A12
VDAC	Voltage-dependent anion channel
VSMCs	Vascular smooth muscle cells

Introduction

Obesity is a significant trigger for various metabolic diseases such as type 2 diabetes mellitus (T2DM), cardiovascular disease, liver failure, and dyslipidemia. Obesity and overweight lead to dysfunction of adipose tissue (Blüher 2013; Saltiel and Olefsky 2017). Adipose tissue is responsible for storing and secreting triglycerides to provide energy during fasting periods, regulate temperature, and protect the mechanical organs. Adipose tissue is classified as an endocrine organ (Vegiopoulos et al. 2017). The release of adipokines as bioactive peptides, lipids, and various metabolites occurs through arrangements of adipocytes, immune cells, and fibroblasts. Adipokines represent a crucial role in regulating inflammatory processes, homeostasis, insulin sensitivity and secretion, energy storage and consumption, and appetite regulation. Modulation of immune cell migration to adipose tissue, adipogenesis, and adipocyte metabolism are the adipokines' main functions in adipose tissues (Fasshauer and Blüher 2015; Francisco et al. 2018). Adipokines affect a variety of biological processes, including adiponectin, leptin, fibroblast growth factor 21 (FGF21), dipeptidyl peptidase 4 (DPP-4), and resistin (glucose metabolism); retinol-binding protein 4 (RBP4), chemerin, and omentin (insulin sensitivity); tumor necrosis factor-α (TNFα), progranulin, resistin, C-reactive protein (CrP), and interleukin (IL)-1b, (IL)-6, and (IL)-10 (inflammation); insulin-like growth factor-1 (IGF-1), fibronectin, and transforming growth factor-β (TGFβ) (growth); adipsin, serum amyloid A3 (SAA3), and acylation-stimulating protein

(ASP) (immune response); and other critical biological functions (de Oliveira Leal and Mafra 2013; Blüher 2014; Booth et al. 2015). In recent years, a visceral adipose tissue-derived serpin, serpin A12 (vaspin), as another adipokine in the adipose tissue-derived serpin subset, has been considered in clinical trials and research studies on diabetes and obesity (Blüher 2012; Salek-Maghsoudi et al. 2019). The focus of this study is on the relationship between type 2 diabetes and vaspin levels. Because type 1 diabetes is an autoimmune disease, pancreatic beta cells are primarily damaged by T cells and cannot produce insulin, resulting in insulin deficiency from childhood or adolescence (Ozougwu et al. 2013). On the other hand, vaspin is a sensitizer of target cells to insulin and has no significant relationship with impaired insulin production. According to recent cell and clinical studies, there is ample evidence of a link between vaspin levels and type 2 diabetes caused by insulin resistance. Overview of current knowledge about the association between vaspin and T2DM through focusing on vaspin as an adipokine and its cellular sources, structure, biochemical function, and, finally, associated disorders is the goal of this chapter.

Type 2 Diabetes and the Importance of Biomarkers

Expanding prevalence of type 2 diabetes (T2D) is classified among predominant medical-related challenges worldwide. It is expected that 592 million people will have T2DM by 2035 (Cui et al. 2018). Insulin insensitivity is a primary hallmark of diabetes called insulin resistance. However, insulin resistance is caused by several factors, and various physiological factors are involved, including genetics, sedentary lifestyle, obesity, unhealthy diet, drugs, pollutants and toxins, and hormonal disturbance (Gutch et al. 2015). All these factors possibly interfere with the secretion of insulin. Innumerable patients of T2DM are identified once multiple complications arose that start soon in the development from normal physiological glucose tolerance to T2DM (Park et al. 2015). This disease not only affects the hormonal balance but also disturbs the standard of living of patients. Diabetes induces burdensome issues such as financial problems, medical charges, medications, lab tests, missed jobs, etc. Due to these issues, diabetes healthcare organizations in the world are bearing huge expenses of billions of dollars every year.

Consequently, persons at risk of T2DM should be identified, and its early diagnosis and management will reduce the onset of disease and its complications (Chatterjee et al. 2017). In order to diagnose and manage a patient with T2DM, multiple laboratory tests are performed. One of the primary diagnostic and monitoring criteria for T2DM is HbA1c and glucose concentrations (Kwon et al. 2015). It has been recognized long ago that more than 50 genes are engaged in the functioning of pancreatic β-cells, the action of insulin, monitoring of glycemic control, or any other conditions (Montesanto et al. 2018). These genes play a vital role in the progression of T2DM, but this concept is controversial because of inconsistent results. Inconsistent results can be due to small sample sizes, distinctive predisposition of T2DM across various radical ethnic communities, dissimilarities in ecological factors, and gene-environment interrelationship. Therefore, some genes were found comprising PPARγ,

KCNJ11, and ABCC8 (Prasad and Groop 2015; Sepand et al. 2021). T2DM is in the category of polygenic disorder. This property is complicated with loci influencing endogenous glucose production, transfer of glucose, and insulin homeostasis which result in challenges to detect diabetes. We can predict T2DM occurrence by considering some biomarkers, which can overcome some of these mentioned limitations (Salek-Maghsoudi et al. 2018; Tans et al. 2019; Maghsoudi et al. 2020).

Vaspin as a Diabetes Biomarker

Vaspin is a member of the serpin subfamily that seems a reliable biomarker among various adipokines involved in diabetes (Escoté et al. 2017). The adipokine (415 amino acids ~47 kDa) was identified in 2000 by Hida et al. in the visceral adipose tissue of an obese type 2 diabetic rat model (Otsuka Long-Evans Tokushima fatty (OLETF)) (Heiker 2014).

Biochemical Characterization

Sequence homology with antitrypsin (about 40%) is the reason for introducing vaspin as part of the serine protease inhibitor (serpin) family. Based on phylogenetic relationships, the serpin superfamily is defined in 16 clades containing 3–77 members (Qi et al. 2017; Motawi et al. 2018). Intracellular and extracellular types of serpins have many functional variations. They have regulatory roles in cellular and inflammatory defense processes, blood coagulation, and protection against ischemia and fibrinolysis (Gatto et al. 2013; Sanchez-Navarro et al. 2020). Vaspin is classified as clade A and serpin A12 (Aibara et al. 2019a). Under natural and active conditions, serpins are monomeric proteins that contain a polypeptide chain consisting of 350–500 glycosylated amino acids (Bhakuni et al. 2016). The primary function of serpins is to inhibit serine proteases. In the serpins' structure, a reactive center loop (RCL) as a pseudosubstrate is exposed to the target protease (Geiger et al. 2015). The amino acid sequence that formed an RCL determines the type of serine protease inhibited by serpins (Marijanovic et al. 2019). The change in serpin conformation occurs due to the protease's binding to the RCL sequence, which ultimately deforms the reactive portion of the protease and inactivates it (Lucas et al. 2018). Vaspin targets proteases that have not yet been fully elucidated, but some recent studies have revealed that kallikrein-related peptidase 7 (KLK7) is one of vaspin's primary targets (Oertwig et al. 2017).

Main Sources of Vaspin

In addition to the presence of vaspin in white adipose tissue, analysis of the tissue expression pattern of vaspin mRNA shows this protein or its mRNA expression in other tissues such as the stomach, liver, pancreas, hypothalamus, and skin (Weiner et al. 2018). In skin tissues, vaspin mRNA expression is prominent. Disruption of the

proteolytic balance between proteases involved in skin tissues and serpins disrupts the integrity and function of the skin's defenses and exacerbates the incidence of inflammatory skin diseases (Shaker and Sadik 2013; Weiner et al. 2018).

Vaspin and Anti-Inflammatory Effects

The primary focus of published studies was on smooth muscle cells and vascular endothelial cells at the cellular level. These studies provided pieces of evidence of anti-atherogenic and anti-inflammatory actions depending on the context of circulatory system disease and obesity-associated inflammation (Phalitakul et al. 2013). Multifold indications demonstrate that vaspin protects endothelial cells from inflammatory components and apoptosis (Phalitakul et al. 2013; Chatterjee et al. 2017; Sepand et al. 2020b). A study illustrated the vaspin inhibition effect of TNF-α-induced adhesion molecule expression in vascular smooth muscle cells (VSMCs). It reduced lymphocyte adhesion through a diminishing generation of reactive oxygen species (ROS) and activation of NF-κβ and protein kinase C (Phalitakul et al. 2011). Additionally, through hindering ROS production along with inhibition of MAP kinase, phosphatidylinositol 3-kinase (PI3K)/AKT, NF-κβ, and insulin receptor signaling pathways, vaspin mitigates high glucose-dependent chemokine production and VSMC expansion (Li et al. 2013; Sun et al. 2015). Likewise, through the deactivation of the PI3K/AKT pathway, vaspin preserves human aortic endothelial cell (HAEC) from apoptosis due to free fatty acids (Jung et al. 2011; Sun et al. 2015; Sepand et al. 2020a). Due to some unique properties, such as creating advanced glycation end products and an extra encouraging element of inflammation and apoptosis, methylglyoxal is a deleterious metabolic product of glucose (Matafome et al. 2013). An investigation displayed that vaspin is responsible for inhibiting ROS generation and methylglyoxal-induced NADPH oxidase activation in human umbilical vein endothelial cells, which subsequently impedes apoptosis-inducing caspase-3 activation (Phalitakul et al. 2013; Salehi et al. 2019). Smooth muscle cell migration, besides inflammatory response, has a vital role in the advancement of atherosclerosis, and vaspin has proven to impede platelet-derived growth factor-BB (PDGF-BB)-induced VSMC migration via suppression of p38 MAPK/Hsp27 signals as well because of decreased ROS generation (Pietruczuk and Srivastava 2017). Further to this, the tone and configuration of vessels are regulated by NO synthase (NOS) and nitric oxide (NO). Hence it is indicated that within the HAEC, vaspin has been considered to enhance NOS action and NO concentration via decreasing the intracellular levels of asymmetric dimethylarginine (ADMA), which is an inhibitor of NOS (Jung et al. 2012; Sato et al. 2018). Such consequences relied on transcription factor STAT3 activation followed by upregulation of gene expression of ADMA-metabolizing enzyme dimethylarginine dimethyl-aminohydrolase. Significantly, not a single study has described the mechanism of vaspin signal transmission from the outside to the inside of the cell. Recent studies demonstrate that vaspin's anti-apoptotic activities within HAEC strongly correlate with a ligand of a membrane confined compound of voltage-dependent anion channel

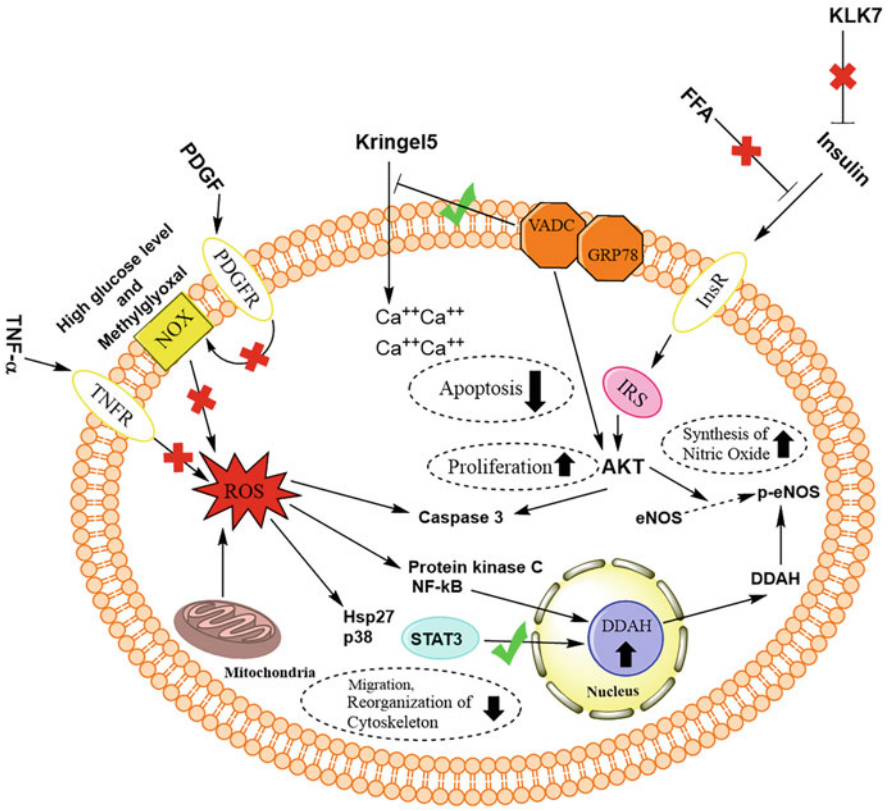

Fig. 1 Vaspin and potential mechanisms associated with diabetes at the cellular level. Anti-apoptotic and anti-inflammatory vaspin signaling pathways: abnormal (red multiplication sign) and inductive (green tick) pathways. TNF receptor, TNFR; insulin receptor, InsR; PDGF receptor, PDGFR; protein kinase C, PKC; NADPH oxidase, NOX; voltage-dependent anion channel, VDAC; insulin receptor substrate, IRS; protein kinase B, PKB; AKT

(VDAC78) and 78-kDa glucose-regulated protein (GRP78) (Nakatsuka et al. 2013; Lin et al. 2016). The GRP78 appears to be a part of vaspin-induced intracellular signaling in H4-II-E-C3 liver cells of rats, suchlike in AKT and AMPK activation (Nakatsuka et al. 2012). However, fundamental molecular processes of vaspin and how vaspin directly acts upon some processes such as adipogenesis, adipocyte differentiation, and adipose tissue function are still unidentified (Fig. 1).

Glucose Intolerance and Vaspin

Since the influence of vaspin gene variants is not well identified, the role of single nucleotide polymorphisms (SNPs) within the chromosome 14 vaspin locus for the progression and advancement of obesity and T2DM is investigated by Kempf et al.

in MONICA/KORA F3 investigation. Conclusions of these studies have demonstrated a significant association between vaspin single nucleotide polymorphism rs2236242 and T2DM with homozygous dominant genotype, which carries a high risk of hyperglycemic disorders. In contrast, this correlation is independent of BMI. Precisely, vaspin and glucose metabolism attain an interconnection, which can be considered an association between obesity and metabolic diseases (especially T2DM) (Kempf et al. 2010). However, there is contradictory evidence regarding serum vaspin levels in T2DM. A study by Ye et al. stated that the patients with T2DM possess elevated levels of vaspin; likewise, their plasma glucose concentrations after eating are positively correlated with vaspin (Yin et al. 2009; Li et al. 2011).

Similarly, it is indicated by Li et al. that continuous subcutaneous insulin infusion causes a decrease in plasma vaspin levels and, at the same time, improves the beta-cell activity in T2DM (Li et al. 2011). It has been anticipated by Jian et al. that reduced concentrations of plasma vaspin levels can be a risk factor for the advancement of T2DM (Jian et al. 2014; Yang et al. 2017b). Obese individuals with prediabetes and normal glucose tolerance (NGT) developed high levels of vaspin (Moradi et al. 2016). While considering the T2DM duration period, plasma vaspin levels have been analyzed in numerous studies. Similarly, Atya et al. stated that the increasing duration of diabetes would be a reduction in vaspin levels (Atya et al. 2013). Another study that included only females diagnosed with diabetes claimed that the increasing duration of diabetes would be a reduction in vaspin levels. There is a significant positive interrelationship between vaspin and HbA1c and BMI and age in the healthy group in both categories, respectively. However, homeostatic model assessment for insulin resistance (HOMA-IR) in T2DM is inversely correlated with different disease durations (Esteghamati et al. 2014; Jian et al. 2014). Based on available resources, possibly it can be concluded that vaspin primarily contributes to the development of T2DM (El-Mesallamy et al. 2011). The assessment of interrelation among plasma vaspin concentrations and the existence of chronic outcomes of T2DM has been done.

In comparison to a poor glycemic control group, female participants of the T2DM group and good glycemic control group indicated reduced vaspin concentrations in a study done by Gulcelik et al., and also the occurrence of microvasculature problems had been introduced to cause low vaspin concentrations (Gülçelik et al. 2009; Auguet et al. 2011). Vaspin plasma concentrations have been measured by Li et al. in participants diagnosed with T2DM for the last 3 years, either accompanying with macroangiopathy or not. Compared to participants with NGT, elevated vaspin levels have been analyzed in T2DM participants without carotid plaques. At the same time, reduced vaspin levels have been studied in T2DM participants with carotid plaques. In participants diagnosed with T2DM for the last 3 years, it has been noted that plasma vaspin concentrations and carotid plaque existence are negatively correlated. Statements mentioned in these studies preconditioned that vaspin might be involved in the mechanism of carotid plaque development in the initial steps of T2DM. As mentioned earlier, the elevated generation of vaspin within adipocytes in that duration possibly acts as a remuneration process linked with obesity, severe insulin

resistance, and progression of diabetes. Due to that reason, we can state that vaspin might act as the latest biomarker and a defensive element for the sake of macrovascular lesions (Li et al. 2012). As demonstrated in various studies, the countervailing ability of vaspin generation slowly reduces with the rise in the period of T2DM or with the beginning of disorders of the heart and blood vessels, leading to a gradual decline in vaspin concentration (Kadoglou et al. 2011; Yang et al. 2017a).

Insulin Role and Vaspin Action

In the last couple of years, after recognizing the KLK7 enzyme to be a target protease, the breakdown of indigenous human insulin with the assistance of KLK7 has been identified. Numerous cleavage sites have been specified inside both chains named A and B, which are compatible or in line with the enzyme restriction sites that metabolize insulin. Co-expression of vaspin and KLK7 within islets of murine demonstrating a potent significance of the vaspin-KLK7 interplay starts due to insulin secretion. Exposing solitary islets with vaspin leads to elevated insulin concentrations within islet supernatants by having a regular and enhanced glucose level while keeping insulin secretion unaffected. The amount of C-peptide remained unaltered (Heiker et al. 2013). Insulin sensitivity and glucose tolerance are reinforced in mice suffering from obesity through administrating recombinant vaspin (Feng et al. 2014). It is also worth mentioning that a persistent glucose decline and reduced food intake resulted from peripheral and intracerebroventricular administration of vaspin (Klöting et al. 2011).

In the current investigation, some mouse strains, such as diabetic db/db mice and C57BL/6NTac, when treated acutely with vaspin, present glucose tolerance, which shows serpin functioning by utilizing the mutant vaspin. The inert mutant utilization could not increase glucose tolerance. Apart from this, elevated insulin plasma concentrations to post-vaspin treatment indicate that suppression of KLK7 due to vaspin is classified among an essential mechanism for its countervailing impact on insulin resistance caused by obesity. This operating principle suggests that the need for vaspin protein increases suddenly after raised blood glucose concentrations and consequential insulin secretion. In a short time, serpin activity decreases in vaspin protein concentration (Heiker et al. 2013; Tindall et al. 2020). A range of studies done on humans supports this broad concept which says that vaspin manifestations accompany a circadian rhythm, so the postprandial peak of insulin secretion causes vaspin rise.

In contrast, acute insulin treatment causes declined vaspin plasma concentration (Kovacs et al. 2013). Despite the fact, within diabetic OLETF rats, administering the insulin chronically causes elevated concentrations of vaspin (Aibara et al. 2019b), but those on insulin treatment display lower vaspin serum concentrations. In white adipocytes of mice, the vaspin mRNA expression improves while treating them with insulin (Heiker 2014). This evidence assists the compensatory theory of declined vaspin serum concentrations following vaspin utilization due to the inhibitory activity of the protease. Limited evidence is available for daily or postprandial

modification in the serum concentrations or expression of KLK7. It was significant to note that the beneficial metabolic impacts of vaspin remain adjusted by vaspin-dependent proteolytic enzymatic pathways that affect glucose consumption and insulin concentrations. Likewise, complexes of an enzyme called serpin are removed from circulation by their receptors and stimulate signaling pathways that improve serpin expression within the cells. But data on vaspin-protease complex receptors are still unknown and undetermined (Hosseini et al. 2011; Dimova and Tankova 2015; Ulbricht et al. 2015; Weiner et al. 2018).

Circulating Vaspin Level, Type 2 Diabetes, and Obesity

Expression of vaspin mRNA in adipocytes was indicated to be linked with T2DM, insulin resistance, and obesity (Shaker and Sadik 2013; Kovacs et al. 2016), whereas, in humans, high vaspin plasma levels are linked with impaired insulin sensitivity and obesity (Feng et al. 2014; Yang et al. 2015). As mentioned earlier, elevated plasma vaspin concentrations in childhood are accompanied by insulin resistance (Yin et al. 2019). Values of average plasma serum concentration in normal individuals are from 0.18 to 1.55 ng/ml. In humans, high vaspin concentrations were correlated with insulin resistance and BMI (Feng et al. 2014; Yang et al. 2017b). In order to identify the association among vaspin plasma concentrations, obesity, and T2DM, Feng et al. conducted a wide-scale meta-analysis. Ultimately, the existence of elevated plasma vaspin concentrations in patients suffering from obesity and T2DM is finalized through these studies (Feng et al. 2014; Rashad et al. 2020).

Metabolic Syndrome (MetS) and Vaspin

Many investigations mentioned that the function of vaspin is vital in the progression of MetS and obesity. In contrast, in these circumstances, it remains unidentified whether it provides protection or not. Manifestation of vaspin mRNA acts potentially as a countervailing approach linked with obesity, progressive insulin resistance, and T2DM. In contrast, any association between plasma vaspin concentration, indicators of insulin sensitivity, and glucose or lipid metabolism remains unidentified. An innumerable number of publications mention that vaspin and MetS variables are positively interrelated (Nicholson et al. 2019). A remarkable positive association occurs between the obesity classes and insulin resistance, with the children (Buyukinan et al. 2018) and the adults (Chang et al. 2010a; Kim et al. 2013) suffering from obesity and T2DM. High vaspin levels have been observed in juvenile Korean males in a cohort study due to some factors such as sedentary lifestyle, obesity, and high immune-reactive insulin concentration during an empty stomach (Han et al. 2013). High serum vaspin concentrations have been measured by Esteghamati et al. in individuals from both genders suffering from MetS. Due to this reason, Esteghamati et al. allocated vaspin as a marker for MetS (Esteghamati et al. 2014). Individuals diagnosed with T2DM and MetS showed no considerable

variation in vaspin serum concentration than a group without MetS (Yan et al. 2014). During elevated HOMA-IR, VAT coordinates with plasma vaspin levels independently, and this association is influenced by insulin resistance (Chang et al. 2010b). It has been indicated that with the existence of metabolic disturbances, insulin resistance and C-reactive protein (being an inflammatory indicator of low intensity) are positively interrelated with vaspin (Karbek et al. 2014; Mogharnasi et al. 2019). Various researches have noted that by weight loss done by modifying the way of living, dietary intake, and physical activity, variations in plasma vaspin concentration have been observed. Individuals with BMI < 25 and with strenuous daily exercise routine showed lower plasma vaspin levels, whereas sudden weight loss due to a complicated exercise routine may lead to high vaspin levels (Kovacs et al. 2016; Shahraki and Eftekhari 2018). It might be supposed that vaspin serves as a responsible agent in the progression of MetS and obesity. During high HOMA-IR, vaspin attains an interrelationship with body mass index, body size and mass, waist size, and hip circumferences (Chang et al. 2010a). Vaspin serum concentrations have been measured by Handisurya et al. in participants with exceptionally obese bodies. They have performed laparoscopic procedures (Roux-en-Y gastric bypass, RYGB) to lose their weight in a short time. Handisurya et al. found a decrease in serum vaspin, leptin hormone, connecting peptide (C-peptide), insulin hormone, body mass index, HbA1c, and HOMA-IR levels, and the alterations in vaspin plasma levels completely interrelate with HOMA-IR, biologically active human insulin, connecting peptide, HbA1c, and leptin. Regardless, the relationship between HOMA-IR and vaspin remains statistically significant even after unification for changes in BMI caused by RYGB (Ibrahim et al. 2018).

Applications to Prognosis, Other Diseases, and Conditions

In this chapter, some large-scale meta-analysis studies and laboratory findings (Feng et al. 2014; Escoté et al. 2017) examining the selection of vaspin as a reliable biomarker in diagnosing and prognosing type 2 diabetes were introduced. Some elderly patients have also shown that vaspin can be considered a potential biomarker in patients with type 2 diabetes and can be used to assess the risk of macromolecular complications (Yang et al. 2017a). Quantitative assessment of the progress of insulin resistance and the quality of function of pancreatic beta cells can be done by using the HOMA-IR method, an acceptable scale for assessing the status of type 2 diabetes (Tang et al. 2015). A recent study verified a positive correlation between vaspin level, HOMA-IR index, and insulin level in patients with type 2 diabetes, confirming the previous studies. Vaspin levels were also significantly higher in patients with T2D and coronary artery disease as a T2D complication (Hao et al. 2016). Another prospective case control study showed that both vaspin mRNA expression and serum levels significantly increased in T2D patients. Another conclusion of this study is that vaspin could be used as a reliable biomarker to assess the risk of ischemic stroke in these patients (Rashad et al. 2020). In contrast, in another study without considering type 2 diabetes, it was found that there is a significant negative

correlation between vaspin level and prognosis and severity of stroke; hence, a decrease in serum vaspin level predicts an increased risk of ischemic stroke (Zhang et al. 2020). One of the most efficient cohort studies performed on more than 1000 patients with acute myocardial infarction (AMI) showed that serum vaspin level as a valid prognostic biomarker could be considered to predict the status of major adverse cardiac events in patients with AMI (Zhou et al. 2019).

Conclusion

In recent years, vaspin as a serine protease inhibitor has been introduced as a molecule with different and beneficial functions in various tissues from the brain to the liver. In this study, the functional mechanism of vaspin was investigated based on its introduction as a potential biomarker of T2D. Laboratory findings in animals and large-scale meta-analysis studies in human populations, overall, showed that vaspin could be considered as a biomarker of T2D. Unfortunately, vaspin cannot currently substitute HbA1c and FGT or other standard parameters used to assess the status of hyperglycemia in T2D. However, according to the available evidence, it is anticipated to have a vaspin test in the diagnostic and prognostic processes associated with T2D in the future.

The vaspin signaling pathway in the context of insulin resistance and T2D is related to the inhibition of proteases implicated in the degradation of proteins involved in balance and reduction of glucose levels or influence on appetite. On the other hand, increased circulating vaspin levels are significantly associated with impaired insulin sensitivity and metabolic syndrome. Furthermore, due to the vaspin presence in different tissues and the possibility of other diseases interfering in intracellular and extracellular vaspin levels changes, more studies are required in the future to select vaspin as a biomarker of T2D.

Mini-Dictionary of Terms

- **Biomarker**. A biological molecule found in body fluids, such as blood, or in various tissues, which specifically distinguishes the normal or abnormal process of the body and the presence, progression, or cure of a disease.
- **Homeostatic model assessment**. It is a method for assessing and quantifying the state of insulin resistance and the function of pancreatic beta cells based on the principle that blood sugar and insulin concentrations are related to the glucose feedback pathway on pancreatic beta cells
- **Kallikrein-related peptidase**. The functional serine protease subfamily with an essential role in regulating normal physiological processes and related pathological conditions, such as immune responses, insulin sensitivity, and glucose metabolism.
- **Serine protease**. Proteolytic enzymes containing serine residues in their active site as amino acids with potential nucleophilic reactions during hydrolysis are involved in various critical physiological processes.

- **Visceral adipose tissue**. A tissue of whole-body fat contains active hormonal components whose secretions have significant biochemical properties in critical signaling pathways of the body. Metabolic disorders such as metabolic syndrome, diabetes, cardiovascular diseases, and cancer are associated with how this tissue serves.

Key Facts of Type 2 Diabetes

- The main difference between type 1 and type 2 diabetes is that in people with type 1 diabetes, pancreatic beta cells cannot produce insulin. However, in type 2 diabetes, the cells' response to insulin is not enough, leading to decreased insulin production.
- Demographically, type 2 diabetes accounts for about 90–95% diabetes cases.
- The development of type 2 diabetes is closely linked to insulin resistance. People with insulin resistance have impaired insulin sensitivity.
- The mechanism of insulin tolerance causes the inefficiency of this hormone. As a result, the body needs an excess of insulin to stimulate muscle and fat cells to take up glucose and the proper functioning of the liver in this pathway.
- In diabetic patients, the prognosis is significantly affected by the level of disease control and its complications. For example, chronic hyperglycemia is associated with an increased risk of microvascular complications.

Summary Points

- Circulating vaspin increases in people with type 2 diabetes.
- From a molecular point of view, vaspin disrupts the serine proteases' functionality, such as KLK7.
- From a cellular point of view, vaspin has anti-apoptotic and anti-inflammatory effects.
- Declined circulating vaspin levels is a sign of progression of type 2 diabetes and its severe and multiple complications.
- Vaspin, along with other diagnostic and prognostic approaches for type 2 diabetes, could be a reliable biomarker in assessing a person's condition.

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Advanced Glycation End Products in Diabetes

7

Jesús A. Mosquera-Sulbarán and Juan Pablo Hernández-Fonseca

Contents

Introduction 173
Advanced Glycation End Products (AGEs) 174
AGE Receptors 175
Effects of AGEs 176
AGE Measurements 177
Effects of AGEs in Diabetes 177
Nephropathy in Diabetes 178
Cardiomyopathy and Arterial Alterations in Diabetes 180
Neuropathy in Diabetes 180
Retinopathy in Diabetes 181
Cancer and Diabetes 182
Cross Talk Between AGEs and Oxidative Stress in Diabetes 183
AGEs in Other Diseases 184
Applications to Other Diseases or Conditions 185
Mini Dictionary of Terms 185
Key Facts of Diabetes: Where Advanced Glycation End Products Is the Topic 186
Summary Points 186
References 187

Abstract

Advanced glycation end products (AGEs) are the product of the interaction of sugars and other glycation inducers with proteins, nucleic acids, and lipids. These compounds have several mechanisms of action. They can alter the biology of organic molecules. Proteins that have been glycated can lose their normal functions and become dysfunctional, as the case of collagen glycation, which can increase the stiffness of the vascular system with consequent dysfunction. AGEs can act on their receptors (RAGE: receptor for advanced glycation end products)

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and activate intracellular signaling pathways leading to nuclear translocation to the transcription factor NF-kB, which induces nuclear activation to produce various inflammatory compounds and oxidative stress. In addition, receptors for AGEs can be stimulated by non-glycated compounds such as S100/calgranulins isoforms, β-amyloid, amphoterin, advanced oxidation protein products, and ligands implicated in vascular injury. These interactions are related to various diseases such as Alzheimer's disease, diseases related to chronic inflammation, and alteration of the vascular system. In diabetes, AGEs induce damage in different systems including the cardiovascular, renal, nervous, and retinal systems mainly mediated by the mechanisms mentioned above. For all these pathological processes involving the AGE/RAGE axis, the use of therapeutic strategies to diminish its effects remains of great interest.

Keywords

Diabetes · Glycation end products · Oxidative stress · NF-κB · RAGE · Hyperglycemia · Renin angiotensin system

Abbreviations

AGEs	Advanced glycation end products
AGE-R1, AGE-R2, or AGE-R3	Advanced glycation end products receptors
Ang II	Angiotensin II
Bcl-2	B-cell lymphoma 2
CKD	Chronic kidney disease
CML	N(carboxymethyl)lysine
CNS	Central nervous system
DR	Diabetic retinopathy
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinases 1 and 2
ERM family	Ezrin, Radixin, and Moesin
GLAP	Glyceraldehyde-derived pyridinium compound
Glo1	Glyoxalase 1
GSK3	Glycogen synthase kinase-3
HIF-1α	*Hypoxia inducible factor-1*alpha
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
LDL	Low-density lipoproteins
LFA-1	Lymphocyte function-associated antigen 1
MAPK	Mitogen-activated protein kinase
NF-Kb	Transcription factor kappa B
NO	Nitric oxide
Nrf2	NF-E2-related transcription factor 2
RAGEs	Advanced glycation end products receptors
RAS	Renin angiotensin system

S-100	Proteins belong to a family of intracellular acidic, low-molecular-weight, and calcium-binding
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule-1
Wnt	Signaling pathways which begin with proteins that pass signals into a cell through cell surface receptors.

Introduction

Advanced glycation end products (AGEs) are formed by the nonenzymatic reaction of sugars and other glycation inducers with proteins, DNA, and lipids. These compounds can be formed by endogenous or exogenous mechanisms. The latter are related to the intake, smoking, and sedentary lifestyle (Cordain et al. 2005; Nursten 2005). AGEs can induce alterations in the organism through mechanisms mediated by the dysfunction of glycated tissues or by inducing inflammatory processes through the production of pro-inflammatory cytokines and oxidative stress (Sessa et al. 2014; Mosquera 2010). AGEs are divided into fluorescent and nonfluorescent compounds. The most important are carboxymethyl-lysine, carboxyethyl-lysine, pyrraline, pentosidine, and methylglyoxal-lysine dimer (Vistoli et al. 2013; Perrone et al. 2020), which have as a common characteristic, the presence of lysine residue. The accumulation of AGEs originates when there is an excess of production and deficit of renal elimination (Vlassara et al. 2008). The accumulation of AGEs and their interaction with its receptors (RAGEs) leads to inflammation, oxidative stress, and endothelial dysfunction (Sessa et al. 2014; Mosquera 2010). RAGEs are multi-ligand molecules expressed in different tissues and cells, which are normally expressed basally but increase during some diseases such as diabetes, cancer, Alzheimer's disease, cardiovascular disease, and aging involving in the progression of those diseases (McRobert et al. 2003; Sessa et al. 2014; Mosquera 2010). In this regard, RAGE activation induces nuclear translocation of NF-kB with consequent nuclear induction of proinflammatory cytokines, adhesion molecules, and oxidative stress (Mosquera 2010; McRobert et al. 2003). There is another group of receptors (AGE-R1, AGE-R2, or AGE-R3) that have opposite effects to RAGE activation related to endocytosis and elimination of AGEs (Xue et al. 2011; da-Cunha et al. 2006; He et al. 2001). RAGE can be found in soluble form probably from the cleavage of RAGE bound to cell membranes; its function is still controversial (Humpert et al. 2006; Prasad 2014). There are several methods for measuring AGEs including high- performance liquid chromatography, mass spectrometry, gas chromatography, and enzyme-linked immunosorbent assay (ELISA) (Röcken et al. 2003; Koetsier et al. 2010; Meerwaldt et al. 2007; Gerrits et al. 2008; Jeong et al. 2016). The most effective way to measure AGEs accumulation is in slow turnover tissue to relate AGEs accumulation to disease progression and pathogenesis. There are new methods for the quantification of AGEs based on monoclonal antibodies that have

been validated (Wendel et al. 2018), but at present there is no gold standard method for the detection and quantification of AGEs. The objective of this review is to describe the scientific evidence on AGEs in relation to their structure, effects on tissues, their association with oxidative stress, and inflammation as well as their effects on the diabetic pathogenesis.

Advanced Glycation End Products (AGEs)

Advanced glycation end products are a complex group of compounds formed by the nonenzymatic reaction of reducing sugars and amine residues in proteins, lipids, or nucleic acids. This reaction, known as the Maillard reaction, produces stable compounds such as AGEs, through the production of unstable compounds such as Shiff bases and Amadori products, which react with peptides and proteins to form protein cross-links (Cordain et al. 2005; Nursten 2005). In vivo the most important AGEs are formed by reacting organic compounds with alpha-dicarbonyl or oxoaldehydes to form N(carboxymethyl)lysine (CML) and pentosidine (Vistoli et al. 2013). AGEs are classified in different groups based on their chemical structures and ability to emit fluorescence (Perrone et al. 2020). In this regard, fluorescent and cross-linked AGEs; nonfluorescent and non-cross-linked; nonfluorescent protein cross-linked and fluorescent non-cross-linked.

The first AGE isolated was pentosidine, formed by collagen where arginine and lysine residues are cross-linked to ribose, hexose, or ascorbic acid (Sell and Monnier 1989). This fluorescent cross-linked AGE represents the major AGE and is used as a measure of the total accumulation of AGEs in plasma and other tissues (van Deemter et al. 2009). CML is one of the most relevant AGEs and is also usually measured as a biochemical marker (Thomas et al. 2018). CML can be formed through various ways. Glucose can bind to lysine residues of the amino group forming Amadori products with subsequent oxidation and CML formation (Hull et al. 2012). Another important component related to AGEs accumulation is pyrraline, which is also formed from the reaction of glucose with lysine residues in proteins (Henning and Glomb 2016). In addition to the cross-linked and fluorescent AGEs, there are the non-cross-linked and fluorescent ones. These bind to the amino acids by their heterocyclic portion, which is replaced by N-H bonds. These compounds act through receptors inducing cellular alterations. An example of these is argpyrimidine, which is formed from arginine and methylglyoxal by a reaction of Maillard (Nemet et al. 2006). Another important AGE inductor is glyceraldehyde 3-phosphate, which is related to GLAP (glyceraldehyde-derived pyridinium) formation, a compound that binds to the lysine and arginine in proteins. This compound is elevated in oxidative stress, inflammation, and diabetes (Tahara et al. 2012). In general, AGEs are very important in vivo, and their accumulation can be measured in pathological conditions such as diabetes (Vlassara and Uribarri 2014).

The origin of AGEs is not only endogenous, but they can also come from food or smoking habit. Prolonged heating of food can generate glyco-oxidation and lipo-oxidation products, which can be ingested. The presence of these AGEs from

smoking and food has been associated with inflammatory events (Cerami et al. 1997; Nicholl et al. 1998). In addition to extracellular AGEs accumulation in tissues and blood, AGEs can also accumulate intracellularly altering protein biochemistry and intracellular signaling pathways (Brownlee 1995).

The circulating levels of ACEs are influenced by the production rate and the ability to be eliminated through various mechanisms. AGEs are metabolized into smaller molecules by the action of glyoxalase 1 (Glo1) in the kidney and eliminated in the urine. Enzyme dysfunction induces accumulation of AGEs in the plasma (Rabbani and Thornalley 2018). There are also compounds such as reduced glutathione, which catalyzes GLAP (glyceraldehyde-derived pyridinium compound) to less toxic compounds such as D-lactate (Xue et al. 2011) and enzymes such as fructosamine kinases, which act by phosphorylating and destabilizing Amadori products (da-Cunha et al. 2006).

AGE Receptors

AGEs exert their effect through three mechanisms: receptor-dependent, non--receptor-dependent, and through their intracellular action. In the receptor-mediated AGE mechanism, there are several proteins that serve as receptors for AGEs such as RAGE, AGE receptors (AGE-R1, AGE-R2, and AGE-R3/galactin-3), and proteins from ERM family (ezrin, radixin, and moesin) (McRobert et al. 2003).

RAGE, a member of the Ig superfamily, is a transmembrane protein that interacts with AGEs inducing activation of protein kinase C and translocation to the nucleus of NF-κB, which induces nuclear transcription of several proteins such as pro-inflammatory proteins, intercellular adhesion molecule-1, E-selectin, endothelin-1, tissue factor, and vascular endothelial growth factor (VEGF) and increases the levels of reactive oxygen species (Sessa et al. 2014; Mosquera 2010). The RAGE-AGE interaction on endothelial cells can activate other additional signaling pathways such as pathways involving nicotinamide dinucleotide phosphate oxidase and MAPKs (Schmidt and Stern 2000), extracellular signal-regulated kinases 1 and 2 (ERK), p21ras, p38, and Janus kinase (Cai et al. 2016). RAGE can bind different types of AGEs and non-AGE proteins such as S-100 isoforms (Stern et al. 2002; Dahlmann et al. 2014), *β*-amyloid related to Alzheimer's disease (Prasad et al. 2019), amphoterin (also known as high mobility group box 1) (Yan et al. 2004; Ando et al. 2018), advanced oxidation protein products (Heidari et al. 2019), and ligands implicated in vascular injury.

RAGE is highly expressed in lungs, skeletal muscle, and heart, but is also expressed in smooth muscle cells, monocytes/macrophages, endothelial cells, astrocytes, and microglia (Ahmad et al. 2018). Basal expression of RAGE increases in pathological conditions such as diabetes, cardiovascular disease, Alzheimer's disease, cancer, and natural aging (Takeuchi and Yamagishi 2009).

A variant of RAGE is the soluble RAGE (sRAGE) that comes from molecular splice of RAGE attached to the cell membrane that has lost the COOH terminal and transmembrane domain and is released to the extracellular medium. This sRAGE can bind to the RAGE ligands in the extracellular medium and prevent the signals

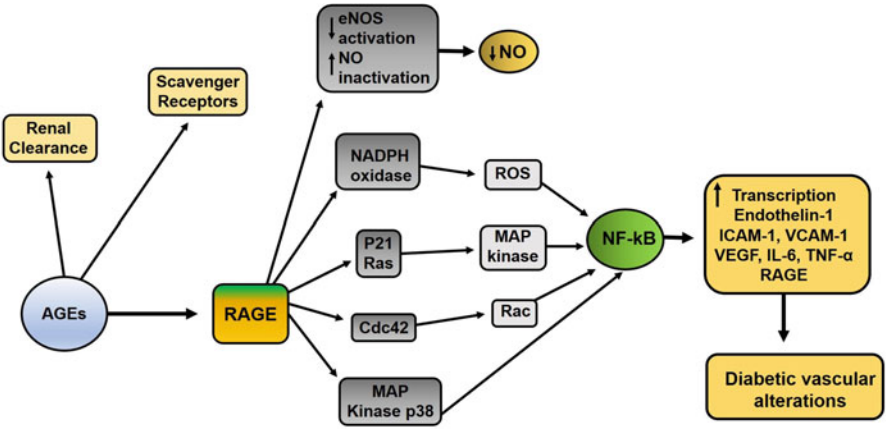

Fig. 1 Effects and receptors of advanced glycation end products (AGEs). AGE receptors are involved in clearance functions (scavenger and clearance receptors) and receptors involved in pro-inflammatory and oxidative stress-inducing effects (RAGE). Cdc42, Cell division cycle 42 protein; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; MAP, mitogen-activated protein; NAD(P)H nicotinamide dinucleotide phosphate; NO, nitric oxide; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-alpha

originated by the interaction of AGEs and other ligands with RAGE. Increased sRAGE levels have been associated with decreased deleterious effects of RAGE activation (Humpert et al. 2006; Prasad 2014).

The AGE-R1 receptor is involved in the clearance and degradation of AGEs. These receptors are present on the surface of macrophages, vascular smooth muscle cells, lung cells, and endothelial cells, and activate scavenging receptor mechanisms (Xue et al. 2011; da-Cunha et al. 2006). Decreased expression of this receptor is associated in humans with increased levels of AGEs and severe complications in diabetes (He et al. 2001). The AGE-R3 receptor belongs to the lectin families and it is overexpressed by hyperglycemia and by the presence of AGEs. Its absence is associated with accumulation of AGEs in the renal glomerulus. The members of the ERM family bind with similar affinity as RAGE to the AGEs (McRobert et al. 2003). The activation of ezrin pathway has been associated with the effect of AGEs on renal tubular cell growth and migration (Gallicchio et al. 2006). Figure 1 summarizes the different AGE receptors: receptors involved in AGEs clearance and receptors involved in pro-inflammatory and oxidative stress-inducing effects.

Effects of AGEs

In general, AGE inducers act by altering the properties and functions of the tissue that they glycolyze or by inducing inflammatory or deleterious processes by interacting with their receptors (RAGE). Previous studies show that AGEs are involved in the pathogenesis of chronic diseases such as diabetes (Maasen et al. 2019), some types of cancers (Walter et al. 2019), neurological and cardiac disorders (Li et al. 2012), among

others. Advanced glycation acts primarily on cell membranes and compounds such as type IV collagen, but proteins such as myelin, tubulin, plasminogen activator 1, and fibrinogen are also prime targets of this glycation (Vlassara 1996). The glycation process of the extracellular matrix (ECM) induces alterations that lead to the resistance of these components to proteolysis and increases stiffness that in the case of collagen and elastin induces blood vessel stiffness (Zieman et al. 2005). In addition, AGEs can induce increased expression of cytokines such as TGF-beta and connective tissue growth factor with increased production of fibronectin, types III, IV, and VI collagen and laminin, modifying the ECM (Throckmorton et al. 1995).

AGEs can interact with their receptors (RAGE) and, in general, produce pro-inflammatory effects. In this regard, AGE/RAGE axis represents an important factor in innate immunity, but it also interacts with endogenous ligands, resulting in chronic inflammation. RAGE signaling has been implicated in multiple human illnesses, including diabetes, atherosclerosis, arthritis, Alzheimer's disease, atherosclerosis, and aging-associated diseases. Interaction of AGEs with RAGE on the cellular surface triggers a series of cellular signaling events, including the activation and translocation to the nucleus of transcription factor NF-kappaB (NF-κB), leading to the production of pro-inflammatory cytokines, chemokines, adhesion molecules, and oxidative stress and causing inflammation (Sessa et al. 2014; Mosquera 2010).

AGE Measurements

Although there are several methods for measuring AGEs, there is no single standard method for their measurement. These compounds have a very complex structure and are difficult to measure them all in a single trial. There are both instrumental and immunochemical methods of measuring ACEs. Among the instrumental methods are: Spectrofluorometer (Röcken et al. 2003), high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS) (Koetsier et al. 2010), gas chromatography coupled with mass spectrometry (GC-MS) (Meerwaldt et al. 2007), liquid chromatography coupled with tandem mass spectrometry(LC-MS/MS) (Gerrits et al. 2008), HPLC with fluorescent detection (Mulder et al. 2006), and method based on ultra-high-pressure liquid chromatography (UHPLC) (Smit and Gerrits 2010). Immunochemical methods are mainly enzyme-linked immunosorbent assay (ELISA) (Jeong et al. 2016) and western blotting (Menini et al. 2018), using antibodies specific for certain AGE structures. However, the selected method must provide reliable information to monitor the health of the patients, the therapeutic effects, and the progression of the studied pathology.

Effects of AGEs in Diabetes

The accumulation of AGEs in different tissues during diabetes is accelerated by hyperglycemia. AGEs form cross-links with the collagen matrix and induce increased rigidity in vessels and myocardium with the consequent alteration of cardiac function (Cooper et al. 2001). In addition, interaction of AGEs with

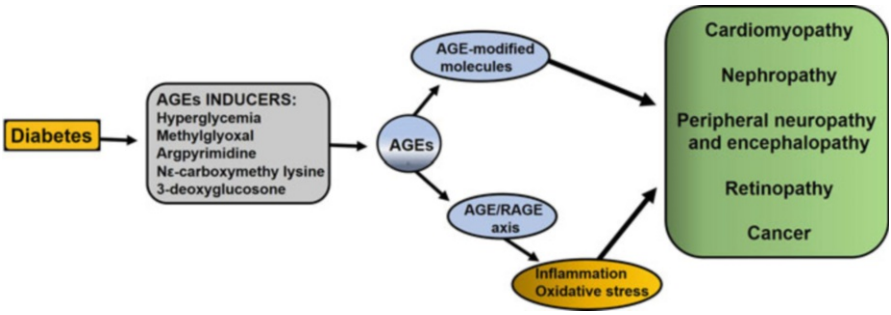

Fig. 2 Mechanisms of action to induce diabetic pathology. During diabetes glycolization inducers form advanced glycation end products (AGEs), which through two mechanisms, alteration of molecules in the tissues and interaction with RAGE receptors, induce pathologies in different organs and systems

RAGE on the cellular surface induces cellular pro-inflammatory signaling events, mediated by activation and translocation to the nucleus of NF-κB and leading to the production of several pro-inflammatory cytokines and oxidative stress, and causing inflammation (Sessa et al. 2014; Mosquera 2010). During diabetes AGEs can affect various organs including the heart, peripheral and central nervous system, kidney, retina, and arteries (Fig. 2).

Nephropathy in Diabetes

High levels of AGEs are associated with uremic cardiomyopathy, and kidney failure in diabetes (Gill et al. 2019). Since the kidney is the major site of AGEs clearance, kidney damage is present during diabetes (Miyata et al. 1998). These compounds contribute to the development of chronic kidney disease (CKD) (Pyram et al. 2012). AGEs usually are metabolized in the kidney into smaller molecules that are subsequently eliminated in the urine. The enzyme that acts on AGEs in the kidney is glyoxalase 1 (Glo1). Its dysfunction induces accumulation of AGEs in the plasma and CKD (Rabbani and Thornalley 2018). Increased blood levels of AGEs induce accumulation of these molecules in the glomerulus promoting increased production of type IV collagen and laminin in the extracellular matrix, inflammation and cell senesce in the proximal tubule. Prevention of diabetic kidney disease by inducing overexpression of Glo1 in transgenic animals has been reported (Rabbani and Thornalley 2018). The pathogenesis of CKD involves adaptive hyperfiltration, AGE synthesis, expressions of prorenin, cytokines, nephrin, and impaired podocyte-specific insulin signaling (Pyram et al. 2012). Mechanisms such as RAS activation and RAGE expression by various renal cells such as podocytes and endothelial cells are involved in renal damage. In this regard, interaction of RAGE with AGEs induces inflammation, glomerular hypertrophy, interstitial fibrosis, and tubular atrophy (Garagliano et al. 2019). These alterations reduce the glomerular

filtration rate and increases the level of circulating AGEs, which their profibrotic activity induces microvasculature damage, accompanying by the actions of the protein kinase C-mediated signal transduction, the RAS activation, and the oxidative stress (Sanajou et al. 2018). These pathological processes have been demonstrated in diabetic animal studies, where the accumulation of AGEs and expression of RAGE in the kidneys is accompanied by functional and structural alterations such as glomerular basement membrane thickening, mesangial expansion, glomerulosclerosis, tubulointerstitial fibrosis (Oldfield et al. 2001), increased collagen IV production, and TGF-β expression (Vlassara et al. 1994). In these experimental studies, diabetic nephropathy is attenuated by ACE-RAGE pathway signaling blockers, such as inhibitors of AGEs formation (Soulis-Liparota et al. 1995), agents that reduce accumulation of AGEs, and RAGE neutralizing antibodies (Flyvbjerg et al. 2004), suggesting the importance of RAGE and AGEs in the induction of renal pathology. For all these renal pathological processes involving the AGE/RAGE axis, the use of therapeutic strategies to diminish its effects remains of great interest (Fig. 3).

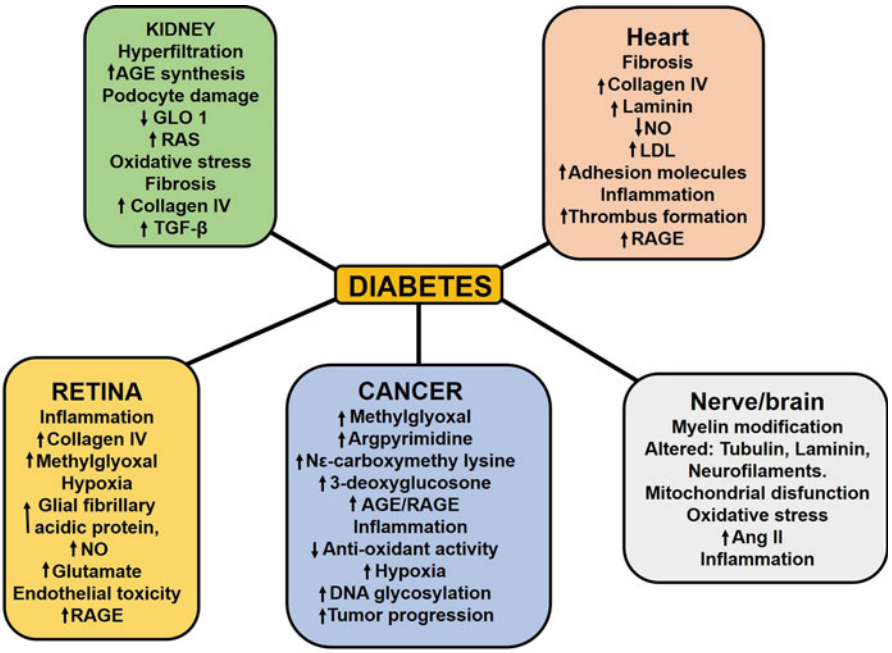

Fig. 3 Alteration of different organs and systems in diabetes. The main organs and systems affected in diabetes are kidney, cardiovascular system, peripheral and central nervous system, retina and predisposition to cancer frequency and mortality. GLO 1, glyoxalase 1; RAS, renin angiotensin system; TGF-β, transforming growth factor beta; NO, nitric oxide; LDL, low density lipoprotein; Ang II, angiotensin II

Cardiomyopathy and Arterial Alterations in Diabetes

The effects of AGEs in the diabetic cardiovascular system are mediated by receptor-dependent and nonreceptor-dependent pathways. In diabetic patients it is common to develop cardiomyopathy, heart failure, alterations in the peripheral arteries and atherosclerosis. This may be related to the cross-linking that AGEs establish with type IV collagen and laminin making the heart and the arteries stiffening and accelerating cardiac fibrosis (Zhao et al. 2014). In this regard, association between high circulating levels of AGEs and increased carotid intima media wall thickness and peripheral artery disease has been found (Lapolla et al. 2007; Yoshida et al. 2005). AGEs are also involved in atherosclerosis through mechanisms such as induction of endothelial dysfunction, increase of low-density lipoproteins (LDL), promoting destabilization of atheromatous plaques by acting on metalloproteinases, inducing proliferation of the vascular intima, and inhibiting vascular repair after injury (Bucala et al. 1994; Sobenin et al. 1993; Kunt et al. 1999). In this regard, the AGEs act by inhibiting nitric oxide and increasing LDL in the subendothelial space, while also preventing LDL clearance by avoiding the interaction of AGE-modified LDL with LDL receptor (Bucala et al. 1994). These events increase atheroma formation due to the accumulation of AGE-LDL in the vascular wall and by the presence of macrophages and foam cells (Sobenin et al. 1993). In addition, AGE/RAGE axis can induce through NF-kB the production of vascular cell adhesion molecule-1 (VCAM-1) with the consequent attraction of monocytes, the first step in atherogenesis (Kunt et al. 1999). In this context, the location of AGEs in atherosclerotic lesions accompanied by macrophages and muscle cells containing lipids in diabetic individuals has been reported (Stitt et al. 1997a). In addition, an association between the degree of accumulation of ACEs and the severity of the atherosclerotic lesion has been demonstrated (Stitt et al. 1997a). Recent studies have shown that binding of AGEs to platelet receptor CD36 induces thrombus formation, a mechanism that may be important in the induction of cardiac ischemia in diabetic patients (Zhu et al. 2012). AGEs can interact with RAS inducing increased proliferation of cardiac fibroblasts and cardiomyocyte hypertrophy in diabetes (Yamazaki et al. 2012). In this regard, experimental studies have demonstrated that Ang II increases RAGE expression in diabetic rat cardiac tissue (Muñoz et al. 2020), and the interaction of AGEs with RAGE can induce NF-kB-mediated overexpression of RAGE (Mahajan and Dhawan 2013) (Fig. 3).

Neuropathy in Diabetes

Diabetic neuropathy is one of the disorders of diabetes that can range from painful manifestations to amputation of limbs. Several mechanisms are involved in diabetic neuropathy mediated by molecular pathways and cytokine production. Among these mechanisms are: polyol pathway, hexosamine pathway, PKCs signaling, oxidative stress, AGEs pathway, PARP pathway, MAPK pathway, NF-κB signaling, hedgehog pathways, TNF-α signaling, cyclooxygenase pathway, interleukins, lipoxygenase pathway, nerve growth factor, Wnt pathway, autophagy, and GSK3 signaling

(Dewanjee et al. 2018). AGEs have several effects related to diabetic neuropathy: the modification of myelin by AGEs makes this protein susceptible to be phagocytosed by macrophages producing demyelination; in the same way, modification of proteins such as tubulin, laminin, and the neurofilament protein can induce nerve atrophy, alterations of nerve regeneration and alterations of axonal nerve transport in diabetes, respectively (Sugimoto et al. 2008). Experimental studies show that AGEs, Glo 1, and mitochondrial diffusion play an important role in diabetic neuropathy (Jack et al. 2012). In this regard, AGE/RAGE axis involved in the development of neuropathy (Suzuki et al. 2020), in the accumulation of AGEs in the skin and in the presence of peripheral small fiber neuropathy during diabetes have been reported (Araszkiewicz et al. 2016). The colocalization of various components of AGE/RAGE axis (accumulation of AGEs, expression of RAGE, NF-κB, and IL-6) in the blood vessels of the peripheral nerves and perineurium has been reported in experimental models of diabetes and human diabetes, suggesting the role of these molecules in the angiopathy and in peripheral nerve neuropathy (Sugimoto et al. 2008; Chen et al. 2004). In addition, AGEs through the induction of oxidative stress can induce diabetic neuropathy in humans (Almogbel and Rasheed 2017).

Complications of chronic diabetes-induced inflammation include peripheral neuropathy; however, this inflammation can be established in the central nervous system (CNS). Diabetes has been implicated as a cause of brain atrophy, white matter abnormalities, and cognitive impairment and a risk factor for dementia (Toth et al. 2007). AGEs and Ang II may act synergistically in the production of diabetic complications (Miller et al. 2013). In this regard, AGEs may induce Ang II expression through mechanisms mediated by the RAGE-PI3-K/Akt-dependent pathway (Cheng et al. 2012). In this regard, experimental studies show inflammatory processes in the CNS mediated by Ang II. In this context, increased expressions of Ang II, ICAM-1, LFA-1, and CD8 positive cells in cerebrum and cerebellum from diabetic rats have been reported; expressions that are decreased by the anti-RAS drugs treatment (Vargas et al. 2012). Light and electron microscope studies from these diabetic rats showed degenerative changes of neurons and glia, perivascular and mitochondrial swelling, disarrangement of myelin sheath, increased area of myelinated axons, presynaptic vesicle dispersion in swollen axonal bottoms, fragmentation of neurofilaments, and oligodendrocyte abnormalities (Hernadez-Fonseca et al. 2009) (Fig. 3).

Retinopathy in Diabetes

Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide, caused by the proliferation and edema (macula) of retinal tissue. Its mechanisms are complex but hyperglycemia, oxidative stress, and inflammation seem to play an important role in the pathogenesis of DR (Sahajpal et al. 2019). Hyperglycemia appears to initiate this pathology accompanied by other pathological processes. In this context, hyperglycemia induces the formation of AGEs that subsequently interact with their receptors (RAGE), activates NF-κB, and increases the cascade of pro-inflammatory molecules (Sessa et al. 2014; Mosquera 2010). In addition, the modification of proteins by

glucose can alter the function of glycosylated tissue (Cooper et al. 2001). AGEs have been shown to accumulate in the collagen of the vitreous humor (Fosmark et al. 2007), and in retinal endothelial cells, neurons and glia (Gardiner et al. 2003; Schalkwijk et al. 1999; Stitt et al. 1997b). One of the main inducers of AGEs in the retina is methylglyoxal and many of the AGEs produced are deposited in the Müller macroglia, which form part of the architecture and physiology of the retina, thus inducing dysfunction and hypoxia in the retinal tissue (Stitt et al. 2002; Agardh et al. 2001). This altered function is manifested by increased production of glial fibrillary acidic protein, NO and glutamate, which is toxic to retinal neurons (Mizutani et al. 1998; Lieth et al. 1998). On the other hand, hypoxia is linked to increased glycolysis through the increment of HIF-1α-dependent glycolytic enzymes and the increment of methylglyoxal synthesis, which contributes to increased AGEs formation in the retina (Van den Enden et al. 1995). The accumulation of AGEs in retinal blood vessels associated with the severity of DR has been documented (Murata et al. 1997; Stitt 2001). However, one of the most reliable methods in the relationship between AGEs accumulation and progression of DR is the measurement of AGEs in proteins with low turnover such as those in the skin (Team 2002). The accumulation of AGEs in retinal tissue is linked to the induction of angiogenesis (small amount) or endothelial cell toxicity (high amounts), leading to capillary closure (Mamputu and Renier 2004; Stitt et al. 2005). Oxidative stress is intimately linked to AGEs formation, and various components of oxidative stress are found in several tissues including retinal tissues, inducing the complications of diabetes such as DR (Kowluru et al. 2001, 2006). The mechanism of how oxidative stress induces retinal AGEs is not clear, but it is known that AGEs can induce oxidative stress, apoptosis, and calcification in retinal pericytes (Chen et al. 2006; Yamagishi et al. 1999, 2012). The involvement of immune processes in the induction of DR has been documented. The expression of adhesion molecules, infiltration of immune cells, and production of proinflammatory cytokines that are probably related to the activation of microglia and infiltrating immune cells have been determined in retinal tissue (Moore et al. 2003; Brucklacher et al. 2008; Joussen et al. 2004; Zeng et al. 2008). The expression of RAGE and various ligands (AGEs, S100/calgranulins, and HMGB1) has been shown to have increased in DR, especially in the glia of inner retina, in the vitreous and preretinal membranes and in Müller cells. The interaction of RAGE with their ligands can trigger inflammatory processes that contribute to DR (Pachydaki et al. 2006; Barile et al. 2005). Experimentally it has been shown that the interaction of ACEs with their receptors promotes the basement membrane thickening of the retinal vessels (Stitt et al. 2000) and increases the expression of the endothelial nitric oxide synthase by endothelial cells (Chakravarthy et al. 1998), which is involved in the vasoregulatory alterations seen in the retinal microcirculation in diabetes (Fig. 3).

Cancer and Diabetes

Previous studies have shown the high risk of cancer in patients with diabetes. The mechanism involved in this propensity is related to the ability of hyperglycemia to glycosylate the extracellular matrix proteins that form the microenvironment of

cancer cells with further interactions with their receptors (RAGE), promoting tumor growth (Rojas et al. 2018). The dependence of tumors on glycolysis induces increased glucose uptake in the tumor microenvironment leading to hyperglycemia, which together with reactive oxygen species leads to the production of AGEs (Eales et al. 2016). Meta-analysis studies show not only the high risk of cancer, but also the high mortality of cancer in diabetic patients and the role of methylglyoxal (a reactive carbonyl species) as a tumor pro-inducer in diabetes (Bellier et al. 2019). Methylglyoxal is mainly formed as a byproduct of glycolysis and, under physiological circumstances, detoxified by the glyoxalase system and is the major precursor of nonenzymatic glycation of proteins and DNA, subsequently leading to the formation of AGEs (Schalkwijk and Stehouwer 2020). AGEs act by binding to their receptors (RAGE) and activating a series of transcription factors with the production of cytokines and inflammatory processes that induce tumor progression (Ahmad et al. 2018). The main glycosylated compounds found in tumors include 3-dG modified DNA, argpyrimidine, and Nε-carboxymethy lysine-modified protein (Heijst et al. 2005). These compounds via RAGE mediate the expression of different genes involved in cancer promotion, such as NOX-2, NF-ķB, SP-1, MMP-2 and -9, Bcl-xl; phosphorylation of ERK 1/2, p38 MAPK, STAT-3, and p70S6K1; down-regulation of Nrf-2 and Bcl-2; p53 expression; and stimulation of PI3K/Akt pathway. All these factors point toward the association of AGEs with the process of malignant transformation via both RAGE-dependent and –independent mechanisms (Palanissami and Paul 2018).

In addition to the implications of the AGE/RAGE axis in tumor progression, protein and DNA glycolization has implications in tumor pathogenesis. In this respect, the glycolization of prohibitin, glycerol-3-phosphate dehydrogenase, lactate dehydrogenase, annexin II, high density lipoprotein, hemoglobin, serum albumin, and histones leads to loss of tumor suppressor function. These alterations consist in elevated carbonyl stress and methylglyoxal levels, impaired redox signaling, and cytoskeletal disorganization (Korwar Arvind et al. 2012), leading to progression and metastasis of cancers (Bing et al. 2012). Enhanced affinity of these compounds to oxygen and hence diminished oxygen supply to tissues result in tissue hypoxia (Jin et al. 2012). Impaired drug-binding affinity and antioxidant activity induce proliferation and invasion of cancers via RAGE binding (Philippe and Emmanuel 2011) and alterations in chromatin structure and genomic instability (Rouf et al. 2015) (Fig. 3).

Cross Talk Between AGEs and Oxidative Stress in Diabetes

The oxidative stress and AGE pathways are not independent and may induce deleterious effects in diabetes (Tahara et al. 2012). The interaction of AGEs with RAGE can lead to the activation of various transduction pathways such as MAPK, ERK1/2, p38, and NF-κB leading to increased reactive oxygen species (ROS), oxidative stress, and deleterious effects on various organs, including the cardiovascular system in diabetes (Zhou et al. 2019). This activation of NF-κB by AGEs also induces the expression of inducible nitric oxide synthase (iNOS) through RAGE/RhoA/ROCK-mediated and AMPK-mediated signaling pathways, and the formation

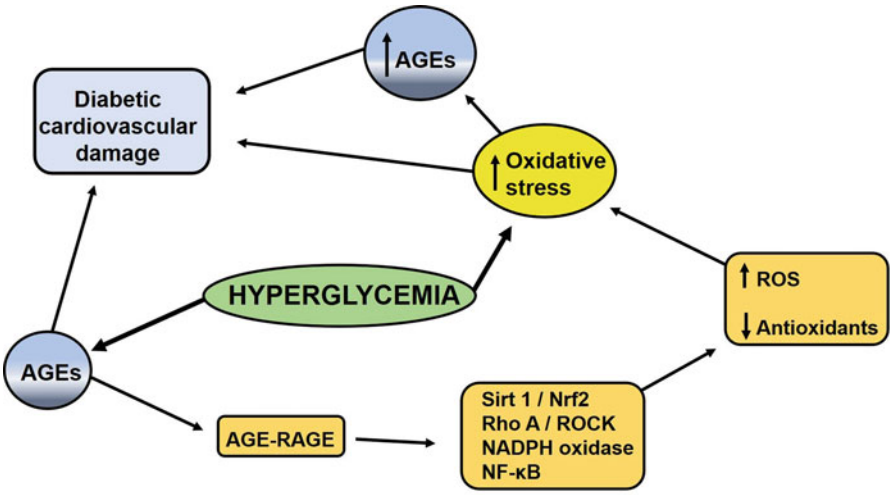

Fig. 4 Advanced glycation end products (AGEs) and oxidative stress

of nitric oxide in endothelial cells (Tang et al. 2016). It has been reported in diabetes that oxidative stress may be also mediated by the Sirt1/Nrf2 axis-induced AGE-RAGE interaction (Chen et al. 2018a). AGEs also induce the activation of NADPH oxidase in endothelial cells with increased ROS and decreased antioxidant compounds such as glutathione, glutathione peroxidase, superoxide dismutase, and catalase, contributing to cardiovascular damage (Chen et al. 2018b*).* In the relationship between AGEs and oxidative stress there is a vicious circle, where AGEs modulate oxidative stress and increased oxidative stress induces increase in AGEs (Chilelli et al. 2013; Dobi et al. 2019) (Fig. 4).

AGEs in Other Diseases

The process of protein glycation has been associated with mechanisms of development of various diseases and complications, such as retinopathy, neuropathy, and nephropathy associated with diabetes mellitus (McCance et al. 1993). The study and quantification of AGEs are also applicable to the analysis of other diseases, thus the mechanisms of action of AGEs are also applicable to these diseases. In this regard, AGEs are involved in diseases such as: macrovascular disease (Vlassara et al. 1992), Alzheimer's disease (Vitek et al. 1994), cataracts (Lyons et al. 1991), and aging (Sell et al. 1996). Histopathological studies and skin autofluorescence analysis have shown that, apart from diabetes, AGEs accumulate in a wide variety of tissues and are mainly associated with conditions of chronic inflammation, including coronary arteries, renal cortex, mesangial and glomerular basement membrane, dermal layer, amyloid plaques in Alzheimer's disease, cartilage in rheumatoid arthritis, cardiac

muscle, lung and liver, lupus erythematosus, and osteoarthritis (Matsumoto et al. 2007; De Leeuw et al. 2007).

These disease complications are directly related to the formation of AGEs and the oxidative stress. The damage produced by hyperglycemia involves complex interactions between the individual's genetics, smoking, body mass index, dyslipidemia, and alterations in coagulation factors (Farouque et al. 2000). The intracellular mechanisms involved in these complications include increased flux of the polyol pathway, activation of protein kinase C, increased hexosamine pathway, and increased AGEs formation. The damage produced by these mechanisms is related to oxidative stress (Brownlee 2001). AGEs lead to the formation of reactive and unstable intermediates that rapidly form intra- and intermolecular covalent cross-links (Grandhee and Monnier 1991) or glycoxidation products (Wells-Knecht et al. 1995). These altered molecules lead to dysfunction of the glycosylated compound, production of pro-inflammatory reactions mediated by AGE/RAGE interactions, and induction of oxidative stress (Banerjee and Chakraborti 2013; Wolff and Dean 1987); these mechanisms interfere with a wide variety of physiological processes that promote atherogenesis (Bucala et al. 1995).

Applications to Other Diseases or Conditions

In this review, the importance of AGEs in the induction of pathology in diabetes was analyzed. One of the highlights was the interaction of AGEs with their receptors (RAGEs) as inducers of inflammatory processes. Although this is a pathology linked to diabetes, other pathological processes may be linked to the interaction of AGEs and other ligands with RAGEs. RAGE can bind different types of AGEs and non-AGE proteins such as S-100 isoforms (Stern et al. 2002; Dahlmann et al. 2014), β-amyloid related to Alzheimer's disease (Prasad et al. 2019), amphoterin (also known as high mobility group box 1) (Yan et al. 2004; Ando et al. 2018), advanced oxidation protein products (Heidari et al. 2019), and ligands implicated in vascular injury. These interactions are linked to diseases such as: macrovascular disease (Vlassara et al. 1992), Alzheimer's disease (Vitek et al. 1994), cataracts (Lyons et al. 1991), and aging (Sell et al. 1996). Hence the importance of analyzing the mechanisms of expression or overexpression of RAGEs and their ligands in these diseases in order to adopt therapeutic measures.

Mini Dictionary of Terms

- **AGEs**. Compounds originated from the interaction of a glycation inducers such as sugars with proteins, lipids, or nucleic acids.
- **AGE/RAGE axis**. Signaling pathway originated from the interaction of AGEs with their receptors inducing inflammatory processes and oxidative stress.
- **Amadori products**. Stable products from the labile Schiff base.

- **Chemokines**. C**hemoattractant cytokines**, which play a vital role in cell migration through venules from blood into tissue and vice versa.
- **Glycated**. Compound chemically linked to a sugar that constitute the AGEs
- **Glycation**. Reaction between a reducing sugar with proteins, lipids, and nucleic acids, where the reactive carbonyl groups of a reducing sugar react with the free amino groups of proteins to form an unstable Schiff base.
- **Glyoxalase 1**. Enzyme that together with glyoxalase 2, and reduced glutathione constitutes the glyoxalase system, which perform an essential metabolic function in cells by detoxifying methylglyoxal (MG) and other endogenous harmful metabolites into nontoxic D-lactate.
- **Maillard reaction**. Chemical reaction between amino acids and reducing sugars involved in AGEs formation.
- **NF-kB**. Transcription factor, which function is crucial in the innate and adaptive immunity by inducing pro-inflammatory cytokines, chemokines, adhesion molecules, and apoptosis regulators.
- **Oxidative stress**. Imbalance between free radicals and antioxidants.
- **RAGEs**. The receptors for advanced glycation end products (AGEs).
- **Reduced glutathione**. Reduced form of glutathione that as endogenous antioxidant plays a major role in reducing reactive oxygen species.
- **Renin angiotensin system**. Hormone system that regulates blood pressure and fluid and electrolyte balance, involved in proinflammatory processes.

Key Facts of Diabetes: Where Advanced Glycation End Products Is the Topic

Diabetes is a very complex and multifactorial metabolic disease characterized by insulin resistance and/or decreased insulin production leading to elevated blood glucose levels.

Hyperglycemia is suggested to be the main cause of diabetic complications,

Sugars and other glycation inducers form advanced glycation end products (AGEs).

AGEs alter the composition of various tissues and their functioning.

AGEs interact with their receptor (RAGE) inducing the production of pro-inflammatory cytokines and oxidative stress.

The main systems affected during diabetes are cardiovascular, neurological, renal, and retina.

Therapeutic approaches to AGE/RAGE axis are important in the treatment of diabetes.

Summary Points

- Advanced glycation end products (AGEs) are formed by the interaction of sugars and other glycation inducers with proteins, nucleic acids, and lipids.

- Proteins that have been glycated can lose their normal functions and become dysfunctional.
- AGEs can act on RAGEs and induce pro-inflammatory status and oxidative stress.
- RAGE activation can be induced by both AGEs and other ligands and is involved in several diseases.
- AGEs can alter different systems in diabetes, which include cardiovascular, renal, nervous system, and retina mainly.
- Blockade of the AGE/RAGE axis represents an important therapeutic approach.

AGEs through interaction with their receptors (RAGE) can induce increase of reactive oxygen species mediated by different pathways and contribute to intensify oxidative stress. Increased oxidative stress can induce the formation of additional AGEs. Both oxidative stress and AGEs can induce deleterious effects on the cardiovascular system in diabetes. Hyperglycemia may contribute to the increase in both AGEs and oxidative stress.

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Markers of Liver Function and Insulin Resistance

8

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Contents

Introduction 196
Applications to Diagnosis 197
Mini-Dictionary of Terms 197
Summary Points (5–10) 198
Markers of Liver Function and Insulin Resistance 199
Visceral Fat and Insulin Resistance 199
NAFLD, IR Y T2DM 200
Hepatokines 201
DPP4 (Dipeptidyl Peptidase-4) 202
Pharmacological DPP-4 Inhibitors 205
Fetuin A (AHSG) 206
Fetuin B 207
Selenoprotein P 207
LECT-2 207
Hepassocin, Also Called Hepatocyte-Derived Fibrinogen-Related Protein 1, HPS, and HFREP1 208
Pigment Epithelium-Derived Factor 209
Tsukushi (TSK) 210
Conclusions 210
References 211

Abstract

Diagnostic features for metabolic syndrome include cardiovascular risk factors, like increased abdominal circumference, triglycerides, and blood pressure, and are mainly characterized by insulin resistance (IR). Moreover, epidemiological and clinical research has shown that non-alcoholic fatty liver disease (NAFLD) is

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continually associated with IR, obesity, type 2 diabetes mellitus (T2DM), and dyslipidemia. Therefore, type 2 diabetes mellitus (T2DM) and NAFLD are studied as two conditions that coexist. However, the relationship between these conditions is so solid and convoluted that it is difficult to identify the consequence and the cause. Further, insulin impairment enhances hepatic glucose production and reduces glucose uptake by peripheral tissues. Thus, NAFLD has been identified as a hepatic expression of metabolic syndrome and is strongly associated with visceral adipose tissue (VAT) and IR. Likewise, molecular mechanisms underlying insulin resistance are influenced by the abnormal secretion of tissue-derived factors such as adipokines, myokines, and hepatokines, which have been recently proposed as praised markers for insulin resistance, NAFLD, and T2DM.

Keywords

Liver function · Insulin resistance · Biological markers · Dipeptidyl peptidase-4 · Ectodysplasin · Fetuin A · Fetuin B · PEDF · Selenoprotein P · Tsukushi

Abbreviations

DPP4	Dipeptidyl peptidase-4
EDA	Ectodysplasin
Fetuin A/B	α-2-HS-Glycoprotein
HFREP1	Hepatocyte derived fibrinogen-related protein 1, hepassocin, or fibrinogen-like protein 1
LECT 2	Leukocyte cell-derived chemotaxin 2, 16-kDA hepatokine, described as chemotactic
PEDF	Pigment epithelium-derived factor
SeP	Selenoprotein P
TSK	Tsukushi, proteoglycan

Introduction

Chronic diseases such as metabolic syndrome, non-alcoholic fatty liver disease, and type 2 diabetes prevalence are continually increasing. These obesity-related metabolic disorders are highly related and share physiopathological components, being the main characteristics of insulin resistance. Moreover, insulin resistance leads to an increased liver fat accumulation, therefore a decisive risk factor for non-alcoholic fatty liver disease due to a rise in free fatty acids' delivery to the liver, triglyceride synthesis, and limited fatty acid oxidation and increased insulin resistance. This condition leads to an incremented liver enzyme delivery and production; some are also proposed as biomarkers of diagnosis and treatment of insulin resistance and obesity-related metabolic disorders. Hepatocyte's protein secretions are known as hepatokines, such as dipeptidyl peptidase-4 (DPP4), fetuin A, fetuin B, selenoprotein P (SeP), hepatocyte-derived fibrinogen-related protein 1 (HFREP1), and tsukushi (TSK), which are increasingly expressed in obese patients with

metabolic impairments. Nonetheless, leukocyte cell-derived chemotaxin 2 (LECT-2) and pigment epithelium-derived factor (PEDF) seem to have a positive correlation with insulin resistance and with metabolic dysfunction-associated fatty liver disease (MALFD), type 2 diabetes mellitus, and obesity. Therefore, they are being targeted as possible biological markers of these conditions.

Applications to Diagnosis

In this chapter, studies related to biomarker levels and liver function in insulin resistance have been reviewed, which show promising use of these markers as alternatives to diagnose insulin resistance, non-alcoholic liver disease, and type 2 diabetes mellitus, proposed to be named as metabolic-associated liver diseases. There is a strong connection between fatty liver and insulin resistance and hepatokine expression in new diagnoses, allowing early identification of these chronic conditions that do not express apparent symptoms. Also, knowing its biological activity leads to the development of drug therapy or lifestyle interventions to enhance or limit hepatokine action, therefore preventing and reducing metabolic impairments.

Mini-Dictionary of Terms

- ***IHTG**, intrahepatic triglyceride, is the intracellular lipid triglyceride accumulation that caused by an imbalance of hepatic fatty acid uptake, lipogenesis, β-oxidation, and triglyceride export.*
- ***PI3K**, phosphatidylinositol-3-kinase, is an enzyme family that participated in diverse cell processes, from cell growth to insulin signaling as immunity and brain development.*
- ***Resistin**, is a cysteine-rich secretory protein that regulates glucose metabolism, also known as an adipokine related to insulin resistance and obesity; in humans is secreted and expressed hormone by macrophages that generated inflammation of adipose tissues through the infiltration of macrophages.*
- ***GLP-1**, glucagon-like peptide-1, is a multifunction incretin hormone derived from the gut, producing the glucose-dependent stimulation of insulin secretion, and decreases inflammation, and apoptosis are some of their effects.*
- ***MALFD** is the abbreviation of metabolic dysfunction-associated fatty liver disease, which describes overweight patients with more additional metabolic disease than hypertension, diabetes, and dyslipidemia.*
- ***JNK**, the c-Jun N-terminal kinase, is a group of MAP kinases, activated generally from cytokines and after cellular damage causing by reactive oxygen species (ROS)contributing to inflammatory responses.*
- ***3 T3-L1** is a cell line that drives 3 T3 cells from mice, under appropriate conditions, has a similar phenotype and appearance as an adipocyte cell, increasing the synthesis and accumulation of triglycerides and being sensitive to insulin drugs, lipogenic and lipolytic hormones.*

- ***ERK1/2****, the extracellular signal-regulated kinases 1 and 2 are part of the mitogen-activated protein kinase superfamily, involved in diverse functions like the regulation of mitosis, meiosis, stimuli cytokines, and growth factors.*
- ***HNF1****, hepatic nuclear factor 1, is a transcription factor with drivers function related to cholesterol, lipoprotein metabolism, and bile acid.*
- ***GLUT-4****, glucose transporter type 4, is an insulin-regulated glucose transporter found in adipose tissues and skeletal and cardiac muscles.*
- ***Key Facts of Insulin Resistance, NAFLD, and T2DM***
  1. *Patients with NAFLD have more than double the risk to develop T2DM than those who do not present NAFLD.*
  2. *Patients with hepatic steatohepatitis have three times the risk to develop T2DM.*
  3. *Hepatokines participate in the regulation of glucose metabolism and insulin sensitivity.*
  4. *The abnormal secretion of tissue-derived factors as adipokines, myokines, and hepatokines from the NAFLD causes insulin resistance.*
  5. *It has been identified that NAFLD is a hepatic expression of MetS and is strongly associated with VAT and insulin resistance.*
- ***Key Facts of Biomarkers of Liver Function Related to Insulin Resistance***
  6. *DPP4 has independent functions from the incretin family, like inducing inflammations and insulin resistance in diverse cellular systems.*
  7. *Selenoprotein P (SeP), fetuin A, and LECT2 have been shown to constrain insulin receptors and enhance the expression of pro-inflammatory cytokines that are involved in insulin resistance.*
  8. *HFREP1 regulates ERK1/2 activity and therefore plays an essential part in insulin resistance development.*
  9. *TSK, a recently established hepatokine, is increased in obese patients.*
  10. *Impairment of PEDF-adipose triglyceride lipase (ATGL) communication can lead to insulin resistance, which has been observed in T2DM patients where PEDF levels were increased.*

Summary Points (5–10)

- *Insulin resistance is strongly related to non-alcoholic fatty liver disease (NAFLD), which is associated with obesity and type 2 diabetes, reaching an average between 50 and 80% of diabetic patients. Furthermore, NAFLD causes insulin resistance in skeletal muscle altered by the impaired secretion of adipokines, myokines, and hepatokines.*
- *Visceral adipose tissue is related to adverse metabolic risk and hypertension, compared to subcutaneous fat, since it is more metabolically active. Therefore, hepatic steatosis is considered a better marker than visceral fat for insulin resistance mediated with obesity.*
- *Adults without insulin resistance have lower liver damage and a reduced prevalence of fibrosis and hepatic steatohepatitis than adults with insulin resistance.*

- *TSK has been shown to reduce plasma high-density lipoprotein cholesterol (HDLc) and cholesterol to bile acid conversion in the liver.*
- *Overweight and obese patients exhibit high plasma concentrations of selenoprotein (SeP).*
- *Hepassocin is increased in diabetic patients with or without hepatic steatosis; high glucose regulates the expression of hepassocin.*

Markers of Liver Function and Insulin Resistance

Insulin resistance (IR) is the main physiopathological feature of metabolic syndrome (MetS), a chronic condition extremely related to non-alcoholic fatty liver disease (NAFLD). Both are recognized for sharing key clinical characteristics. Likewise, diagnostic features for metabolic syndrome include cardiovascular risk factors, like an increased abdominal circumference, triglycerides, and blood pressure. For NAFLD diagnosis, histological images and ultrasound examinations are required to identify liver steatosis or cirrhosis (Bril et al. 2017). Both metabolic syndrome and NAFLD are classified as metabolic disorders with a high prevalence and represent a global public health burden. In Western countries, the prevalence reaches 20–30% of adults and an average of 80% in patients with diabetes or obesity. Data from the National Health and Nutrition Examination Survey of the United States shows that metabolic syndrome is diagnosed in over 30% of the adults, in Europe reaches 24.3%, and in China, according to a systematic review, MetS prevalence is present in 24.5% of people over 15 years (Cao et al. 2021). Epidemiological and clinical research has shown that NAFLD is continually associated with insulin resistance, obesity, diabetes, and dyslipidemia (Cheung and Sanyal 2010).

Obesity is linked with both conditions since increased waist circumference is a risk factor for metabolic syndrome and may be related to fat accumulation on visceral tissue, particularly the liver, leading to NAFLD. However, not all obese adults develop metabolic disorders; some are described as metabolically healthy obese; although there is no unified definition, some consider one or two risk factors for MetS. The exact mechanisms that protect their metabolic health have not been yet elucidated. Even without insulin resistance, obese patients are advised to lose weight; further analysis is required to understand the progress of obesity towards other metabolic disorders (Engin 2017).

Visceral Fat and Insulin Resistance

Since the early 1990s, the link between IR, reduced high-density lipoprotein cholesterol (HDL), and glucose intolerance has been observed. Therefore, MetS seem to worsen NAFLD and increase the risk to develop liver steatohepatitis (NASH) and other comorbidities. The main factor of this condition is related to increased lipolysis and increased hepatic lipogenesis (Engin 2017). Furthermore, rise in free fatty acid concentration leads to an intrahepatic fat accumulation through activation of

hormone-sensitive lipase (HSL), responsible for reducing insulin to receptor binding, due to lipotoxicity, reducing insulin receptors on targeted tissues (Liu et al. 2020). Therefore, insulin impairment enhances hepatic glucose production and reduces glucose uptake by peripheral tissues (Lan et al. 2014), therefore compromising the primary source of glucose uptake mediated by insulin, since around 40% of total body mass is the skeletal muscle (Myers et al. 2019). However, genetic and epigenetic factors are involved in metabolic syndrome and NAFLD. Also, diet, lifestyle, oxidative stress, and microbiota may be interconnected as synergy in the hepatocyte that leads to liver damage (Engin 2017).

On the other hand, it has been suggested that waist circumference and body mass index (BMI) may be the most accurate alternative of markers for IR, visceral adiposity, and hepatic steatosis in youth (Borruel et al. 2014). Hepatic steatosis is considered better than visceral fat as a marker for multiorgan insulin resistance mediated with obesity (Jung et al. 2018a). Increased visceral adipose tissue (VAT) shows higher concentrations of soluble tumor necrosis factor-alpha receptor 2 (TNF-alpha) (Janiszewska et al. 2021). Thus, VAT remains notably associated with an adverse metabolic risk for IR and hypertension, compared to subcutaneous fat, since it is more metabolically active (Engin 2017; Rochlani et al. 2017). In addition, VAT synthesizes significantly higher amounts of bioactive secretory proteins such as plasminogen activator inhibitors, promoting a prothrombotic state (Rochlani et al. 2017). Also, it has been shown that adults without IR have lower liver damage and a reduced prevalence of fibrosis and hepatic steatohepatitis than adults with insulin resistance (Acierno et al. 2020).

NAFLD, IR Y T2DM

According to a recent experts' consensus, replacing the acronym "NAFLD" with "MALFD," "metabolic-dysfunction-associated fatty liver disease," has been proposed for being considered as a more accurate term that includes the diversified and convoluted causes and the broad spectrum of clinical complications, as well as considering inter-patient differences (Eslam et al. 2020).

NAFLD has been identified as a hepatic expression of MetS and is strongly associated with VAT and insulin resistance. Even prevalence of NAFLD was over 50% in diabetic adults with obesity (Portillo-Sanchez et al. 2015). Therefore, type 2 diabetes mellitus (T2DM) and NAFLD are studied as two conditions that coexist (Marchesini et al. 2016). More than 70% of T2DM patients also develop NAFLD; moreover, almost 20% of diabetic adults also present hepatic fibrosis (Acierno et al. 2020).

Patients with NAFLD have more than double the risk of developing T2DM than those who do not present NAFLD (Mantovani et al. 2020), and patients with hepatic steatohepatitis have three times the risk to develop T2DM (Chalasani et al. 2012; Marchesini et al. 2016). The connection between both conditions is robust, convoluted and difficult to differentiate between the cause or the consequence (Acierno et al. 2020). NAFLD is accompanied by a vast spectrum of conditions that goes from

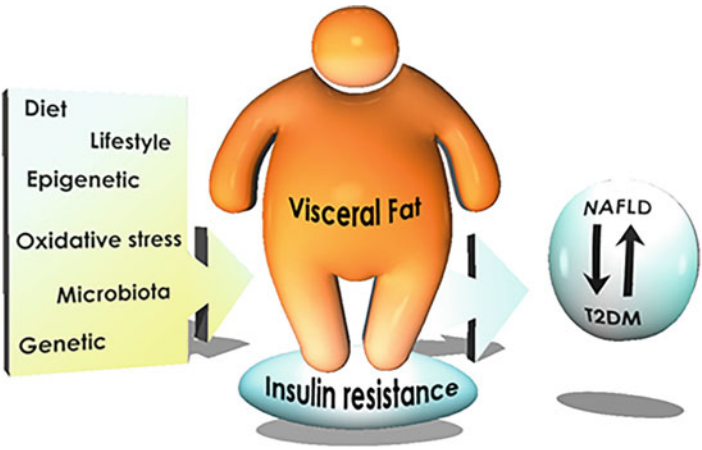

Fig. 1 Relationship between visceral fat with insulin resistance. Visceral fat accumulation results from a complex variety of risk factors such as high-fat diet, lifestyle like sedentary behavior, epigenetic, and also the increase of oxidative stress; a dysbiosis of the microbiota and genetic factor are involved in the increased risk of NAFLD and T2DM mediated trough insulin resistance and VAT, since it is more metabolically active than subcutaneous fat. Moreover, increased VAT shows higher soluble TNF-alpha concentrations, which is strongly associated with an adverse metabolic risk for IR. VAT = visceral adipose tissue; TNF-alpha = tumor necrosis factor-alpha receptor 2; IR = insulin resistance; NAFLD = non-alcoholic fatty liver disease; T2DM = type 2 diabetes mellitus

a relatively benign intrahepatic triglyceride (IHTG) accumulation to non-alcoholic steatohepatitis (NASH), cirrhosis, and an increased risk of liver cancer (Friedman et al. 2018). Furthermore, impairment of glucose tolerance is present in an average of 70% of patients with liver cirrhosis (Acierno et al. 2020).

Both chronic conditions, NAFLD and T2DM, hold several physiopathological features involved in the evolution of the disease, along with a high level of free fatty acids, pro-inflammatory cytokines, and oxidative stress (Fig. 1).

Hepatokines

Hepatokines are described as liver-secreted proteins exclusively expressed by hepatocytes, which have been recently identified as part of the endocrine system (Table 1), improving or worsening metabolic conditions (de Oliveira Dos Santos et al. 2021). The mechanisms behind IR are acknowledged to be altered by secretion impairment of adipokines, myokines, and hepatokines (Lan et al. 2014). Hepatokines regulate glucose metabolism and insulin sensitivity and other tissue-derived factors such as adipokines (Mohri et al. 2019). Moreover hepatokines have an endocrine-dependent relationship with other tissues, acting via crosstalk with the cytokines released by adipocytes and skeletal muscle (de Oliveira Dos Santos et al. 2021). Preclinical, mechanistic studies show hepatokines are related to long-term

Table 1 Liver biomarkers and functions

Hepatokines	Functions
DPP4	Induce insulin resistance and inflammation process (Shao et al. 2020), correlate with lipotoxicity-induced liver damage and macrophage M1/M2 status (Balazki et al. 2020). DPP4 enhances adipose inflammation through PAR2- and TLR4-mediated pathways and insulin resistance (Ozcan et al. 2021)
Fetuin A	Inhibits the insulin receptor tyrosine kinase (Olivier et al. 2000) and downregulates the expression of adiponectin (Bourebaba and Marycz 2019)
Fetuin B	Promotes insulin resistance development (Qu et al. 2018).
SeP	Antioxidant enzyme, delivers selenium to relevant tissues (Misu 2019)
LECT-2	Natural killer T cell homeostasis and hepatic inflammatory signaling (Jung et al. 2018b)
HPS	Regulation c of hepatocyte proliferation and liver regeneration (de Oliveira Dos Santos et al. 2021), associated to fasting glucose, insulin resistance, and T2DM (Ou et al. 2015) Regulates mitogen-activated protein kinase ERK1/2 activity (Wu et al. 2016)
PEDF	Anti-angiogenic, anti-proliferative, neurotrophic, and immunomodulatory activity (Maeda et al. 2011)
TSK	Energy expenditure regulation and adipose tissue thermogenesis (Xiong et al. 2019)

Functions of liver biomarkers (DPP4, fetuin A, fetuin B, SeP, LECT-2, HPS, PEDF, and TSK) are described. DPP4 = dipeptidyl peptidase-4; M1/M2 = macrophages classically activated/macrophages alternatively activated; PAR2 = protease-activated receptor 2; TLR4 = Toll-like receptor 4; SeP = selenoprotein P; LECT-2 = leukocyte cell-derived chemotaxin 2, 16-kDA hepatokine, described as chemotactic; HPS = hepassocin; T2DM = type 2 diabetes mellitus; ERK1/2 = extracellular signal-regulated kinases 1/2; PEDF = pigment epithelium-derived factor; TSK = tsukushi

energy balance; thus, chronic high-fat overfeeding modulates hepatocyte gene expression (Willis et al. 2020) (Fig. 2).

Altered hepatokine expression and secretion have been observed in NASH inducing diet-fed mice and patients with NAFLD (Meex and Watt 2017). Also, high mobility group box 1 protein (HMGB1), a hepatokine, has recently shown a positive correlation with homeostatic model assessment for insulin resistance (HOMA-IR) and be raised in subjects having high fasting blood glucose. Therefore, HMGB1 blocking therapy is suggested as a possible treatment to reduce the inflammatory process; thus, HMGB1 inhibition will improve all components of metabolic syndrome (Nasir et al. 2020). Furthermore, based on hepatokine studies, therapies using DPP-IV inhibitors (gliptins) have been developed for T2DM management (Wasana et al. 2020). Finally, hepatokines are a promising field as biomarkers and future targets for treatment in metabolic disorders (Table 2).

DPP4 (Dipeptidyl Peptidase-4)

DPP4 is a serine protease that cleaves various substrates, including incretin hormones, chemokines, growth factors, and neuropeptides. It has been shown to act in an incretin-independent manner, inducing IR and the inflammation process (Shao

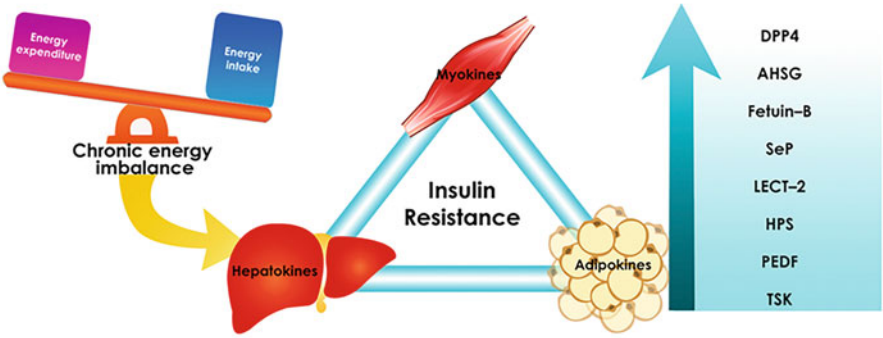

Fig. 2 Insulin resistance and its relationship with hepatokine and adipokine biomarkers. Hepatokines are described as liver-secreted proteins exclusively expressed by hepatocytes, which have been recently identified as part of the endocrine system, improving or worsening metabolic conditions. The mechanisms behind IR are acknowledged to be altered by secretion impairment of adipokines, myokines, and hepatokines. Hepatokines participate in regulating glucose metabolism and insulin sensitivity and other tissue-derived factors such as adipokines. Moreover, hepatokines are related to long-term energy balance; thus, chronic high-fat overfeeding modulates hepatocyte gene expression, leading to increased secretion of DPP4, AHSG, fetuin B, SeP, LECT-2, HPS, PEDF, and TSK. DPP4 = dipeptidyl peptidase-4; AHSG = fetuin A; SeP = selenoprotein P; LECT-2 = leukocyte cell-derived chemotaxin 2, 16-kDA hepatokine, described as chemotactic; HPS = hepassocin; PEDF = pigment epithelium-derived factor; TSK = tsukushi; IR = insulin resistance

et al. 2020). DPP-4 activity is correlated with lipotoxicity-induced liver damage and macrophage M1/M2 status (Balazki et al. 2020).

Preclinical and clinical studies have shown that hepatic DPP4 enhances adipose inflammation through protease-activated receptor 2 (PAR2)- and Toll-like receptor 4 (TLR4)-mediated pathways and insulin resistance (Ozcan et al. 2021). DPP4 is a significant contributor to NAFLD development in a high-fat diet-induced metabolic impairment model (Baumeier et al. 2017b). An increased expression of hepatic DPP4 results in IR and significant liver steatosis in mice; thus, DPP4 have been presented as an accurate marker for hepatocyte apoptosis and fibrosis. Likewise, obese patients with IR shows a DPP4-dependent inflammatory activity that was not observed in obese subjects without IR (Ghorpade et al. 2018).

The American Diabetes Association and the European Association for the Study of Diabetes **suggest** dipeptidyl peptidase-4 inhibitors (DPP4i) as promising **combination treatment in new-onset cases of T2DM** and second-line therapy after metformin in subjects with lessen cardiovascular risk (Angwin et al. 2020; Buse et al. 2020). Further, DPP4i are associated with a lower risk of hypoglycemia, may delay the progression of albuminuria (Ogundipe et al. 2021), and has a favorable long-term safety and tolerability, being nasopharyngitis and skin lesions the principal potential adverse effects, being the risk of pancreatitis lower (Franco et al. 2021).

Additionally, preclinical studies in human adipocytes showed that administration of DPP4 resulted in insulin resistance, though DPP4 reduction improved insulin sensitivity (Baumeier et al. 2017b). Therefore, based on DPP4 activity, the use of

Table 2 Hepatokines and promising activity as biomarkers and therapeutic targets

Hepatokines	Description	Promising activity
DPP4	Major contributor in NAFLD development in a high-fat diet-induced metabolic impairment model (Baumeier et al. 2017b)	Accurate maker for hepatocyte apoptosis and fibrosis. Additionally, patients with obesity show DPP4 dependent inflammatory activity (Ghorpade et al. 2018) DPP4 reduction improves insulin sensitivity (Baumeier et al. 2017b)
Fetuin A	Hepatokine is related to obesity, liver fat accumulation, and endothelial dysfunction (Sindhu et al. 2016) Decreased levels are associated with weight loss and fatty liver reduction (Zhang et al. 2018)	Levels are increased in patients with MetS and T2DM (Ren et al. 2019). Moreover, higher levels of fetuin A are related to retinopathy development (Yilmaz et al. 2018) Promising marker for T2DM incidence (Esfahani et al. 2019)
Fetuin B	The second member of the fetuin family is reduced during the acute inflammation phase in a rat model (Olivier et al. 2000)	Can be an independent predictor for NAFLD in patients with T2DM (El-Ashmawy and Ahmed 2019) Exhibits negative association with adiponectin concentration in patients with T2DM (Tsutsumi and Saito 2020)
SeP	Rich in selenocysteine glycoprotein, secreted and primarily expressed by the hepatocytes (Polyzos et al. 2020)	Increased levels have been found in patients with glucose impairment (Mohri et al. 2019)
LECT-2	Energy-sensing hepatokine upregulated in response to overnutrition (Willis et al. 2020)	Biomarker to elucidated hepatic steatosis related to IR (Jung et al. 2018b)
HPS	Obesity-induced hepatokine, capable to promote insulin resistance (Jung et al. 2018a)	Is increased in patients with pre-diabetes, T2DM, and NAFLD (Wu et al. 2016). Is proposed as a potential target for the treatment of obesity-linked IR and T2DM (Jung et al. 2018a) Biomarker for analyzing risk states for T2DM (de Oliveira Dos Santos et al. 2021)
PEDF	Most abundant protein secreted by adipocytes; thus, PEDF-ATGL impairment can lead to insulin resistance (Huang et al. 2018).	Independently associated with fasting ApoB8 levels, implying that PEDF concentration may be a biomarker for postprandial hyperlipidemia (Tahara et al. 2012) PEDF values are proposed as markers and potential therapeutic targets of coronary artery inflammation in T2DM patients (Tahara et al. 2020)

(continued)

Table 2 (continued)

Hepatokines	Description	Promising activity
TSK	Leucine-rich proteoglycan, recently identified as hepatokine (Wang et al. 2019).	Increased in obesity (de Oliveira Dos Santos et al. 2021) Related to liver fat accumulation (Mouchiroud et al. 2019b)

Hepatokines such as DPP4, fetuin A, fetuin B, SeP, LECT-2, HPS, PEDF, and TSK have a promising activity as biomarkers and therapeutic targets. DPP4 = dipeptidyl peptidase-4; NAFLD = non-alcoholic fatty liver disease; MetS = metabolic syndrome; T2DM = type 2 diabetes mellitus; SeP = selenoprotein P; LECT-2 = leukocyte cell-derived chemotaxin 2, 16-kDA hepatokine, described as chemotactic; IR = insulin resistance; HPS = hepassocin; PEDF = pigment epithelium-derived factor; PEDF-ATGL = pigment epithelium-derived factor-adipose triglyceride lipase; TSK = tsukushi

inhibitors as therapy for NAFLD patients have been proposed to enhance insulin sensitivity and limit the further accumulation of intrahepatic fat (Baumeier et al. 2017a). Additionally, DPP4i have complex metabolic activity and modifies the metabolism regulatory peptides and chemokines (Beaudry and Drucker 2020).

Pharmacological DPP-4 Inhibitors

Drugs with DPP-4 inhibitory activity are also very effective and widelyzed in treating T2DM (Abubakar et al. 2021). In addition, it has been shown that gemigliptin, a DPP4i approved in more than ten countries worldwide, can be safe to use in patients with renal insufficiency in both moderate and severe cases. Also, a recent meta-analysis provides information to support its favorable glycemic efficacy and tolerability over 6 months of clinical use (Dutta et al. 2021).

Additionally, DPP4i with insulin improved glycemic control (Alsalim et al. 2020) without increasing the risk of hypoglycemia or weight gain compared with insulin treatment alone (Yang et al. 2018). Also, a combination of metformin and DPP4i treatment has been found to be cost-effective compared to a combination of metformin and sulfonylureas as a long-term second-line treatment (Know et al. 2018). Combination therapy of DPP4i and glucagon-like peptide-1(GLP-1) receptor agonists has shown benefits in gestational diabetes mellitus (GDM). However, more clinical trials are required to recommend it as therapy for GDM patients (Chen et al. 2020). Between DPP4i, \anagliptin has been shown to increase the relative proportion of M2 to M1 macrophages in the liver, thus preventing insulin resistance and fibrogenesis, which suppress steatohepatitis development in mice (Sakai et al. 2020). Moreover, other DPP4i, trelagliptin succinate, promotes glucose intake via stimulation of GLUT4 translocation in adipocytes and limits the secretion of free fatty acids and resistin in rat adipocytes (Liu et al. 2020).

In patients who do not respond to insulin therapy, adding a DPP-4 inhibitor can reduce HbA1c levels without increasing hypoglycemic incidence (Shibuki et al. 2020). Also, moderate restriction of carbohydrates decreases HbA1c levels among Japanese patients with T2D, treated with DPP-4 inhibitors (Kobayashi et al. 2020). Moreover, recent studies have shown that some bioactive peptides in pork meat have DPP4-inhibitor activities; these may lead to the development of functional products to be part of prevention or treatment-adjuvant for T2DM patients (Kęska et al. 2019).

The efficacy of DPP4i clinical use is determined by the degree of patients' adherence to treatment (Ogundipe et al. 2021). Likewise, it is interesting that only improvement in liver function tests was significantly associated with lower DPP-4 in the weight loss cohort (Ozcan et al. 2021).

Fetuin A (AHSG)

Fetuin A has been described as an important protein along with fetal life, and it takes part in relevant functions such as inhibiting the activity of insulin receptor tyrosine kinase (Olivier et al. 2000) and may have a role in macrophage accumulation in the islets. However, further research is needed to support this role (Mukhuty et al. 2017).

The increase in fetuin A levels has shown a positive correlation with obesity (Goustin and Abou-Samra 2011), liver fat accumulation, endothelial dysfunction (Sindhu et al. 2016), and atherosclerosis development and exhibits a negative relation to insulin-sensitivity due to a downregulated expression of adiponectin (Bourebaba and Marycz 2019). Therefore, several studies have focused on the link between fetuin A concentrations and the risk of T2DM (Mori et al. 2006). Circulating increased concentrations of fetuin A have been reported in MetS (Ren et al. 2019) and T2DM. A recent meta-analysis has shown that T2DM patients exhibit higher fetuin A levels compared to non-diabetic individuals. Moreover, non-diabetic patients with high fetuin A levels exhibit more than 20% higher risk of developing the condition (Roshanzamir et al. 2018). Additionally, in diabetic patients, the risk of retinopathy development increases with higher fetuin A values, therefore playing an important role in the pathophysiology and progression of diabetic retinopathy (Yilmaz et al. 2018). This hepatokine is described as a promising marker for T2DM incidence after adjusting risk factors (Esfahani et al. 2019).

On the other hand, some interventions have been shown to reduce fetuin A levels; from a pharmacological perspective, liraglutide exhibits a major reduction in the visceral adiposity volume and fetuin A than pioglitazone in patients with T2DM (Zhang et al. 2020) and NAFLD (Esfahani et al. 2019); also, pioglitazone lowers mRNA expression of fetuin A in mice (Zhang et al. 2018). Also, decreased fetuin A levels are associated with weight loss and reduction of fatty liver (Zhang et al. 2018). Thus, regular exercise improves whole-body and liver insulin sensitivity (Ennequin et al. 2019) due to a reduction in fetuin A levels and caloric restriction (Esfahani et al. 2019).

Therefore, fetuin A has been proposed as an accurate target for developing of T2DM treatment strategy (Esfahani et al. 2019).

Fetuin B

Fetuin B is the second member of the fetuin family, is found in humans and rodents, and is primarily produced by the liver. Like fetuin A, fetuin B level is reduced during the induced inflammation acute phase in a rat model (Olivier et al. 2000). Fetuin B can promote insulin resistance in myotubes and hepatocytes and caused glucose intolerance in mice (de Oliveira Dos Santos et al. 2021).

T2DM and NAFLD patients showed significantly raised plasma fetuin B, and increased circulating fetuin B raises fasting insulin level, which results in insulin resistance (Qu et al. 2018). Therefore, fetuin B links NAFLD to T2DM (Meex et al. 2015) by promoting insulin resistance (Lin et al. 2018). Thus, fetuin B concentration can be an independent predictor for NAFLD in patients with T2DM (El-Ashmawy and Ahmed 2019).

Selenoprotein P

Selenoprotein P (SeP) is a selenocysteine glycoprotein secreted and primarily expressed by the hepatocytes and affluently expressed in human blood (Polyzos et al. 2020). SeP acts as an antioxidative enzyme, thus limiting oxidative stress and delivering selenium to relevant tissues (Misu 2019).

SeP concentrations are elevated in patients with glucose impairment (Mohri et al. 2019) and were related to various cardiometabolic features, including insulin resistance, inflammation, and aggravating the risks of atherosclerosis (Yang et al. 2011). Also, the plasma concentration of SeP exhibits a negative association with adiponectin (Tsutsumi and Saito 2020) concentration in T2DM patients (Esfahani et al. 2019). Therefore, high concentrations of SeP play a detrimental role in inducing IR and hyperglycemia in pre-diabetes and T2DM (de Oliveira Dos Santos et al. 2021). Additionally, overweight and obese patients show high plasma concentrations of SeP and usually exhibit lower adipocytes expression of SeP. However, after adjustment with BMI, there was no association, implying that adiposity may be the leading driver in this connection; nonetheless, further studies in more extensive, controlled cohorts are required (Chen et al. 2017).

Contrary to results in T2DM patients, clinical studies have shown lower concentrations of Se and SeP in patients with cirrhosis or hepatic carcinoma than in controls; however, recent evidence is not yet enough to recommend Se or SeP for diagnostic nor therapy for NAFLD patients since it is a multifactorial condition which required a multi-targeted therapeutic approach (Polyzos et al. 2020).

LECT-2

Leukocyte cell-derived chemotaxin 2 (LECT2) is a recently discovered hepatokine and secretory protein identified as a novel neutrophil chemotactic protein (Lan et al. 2014), expressed by the liver (Misu 2018). In addition, it is an energy-sensing

hepatokine upregulated in response to overnutrition (Willis et al. 2020). Moreover, it has been related to hepatic inflammatory signaling and natural killer T cell homeostasis (Jung et al. 2018b).

An experimental rodent model has exhibited that LECT2 improves insulin sensitivity in skeletal muscle, although overproduction of LECT2 contributes to muscle insulin resistance in obesity (Lan et al. 2014). LECT2 knockout mice improve insulin sensitivity implying its role in metabolic disorders (Lebensztejn et al. 2016). Also, LECT2 increases mammalian target of rapamycin (mTOR) phosphorylation, lipid accretion, and IR in human liver cancer cells (Esfahani et al. 2019). It has been shown that increased LECT2 production may contribute to the early reduction in whole-body insulin sensitivity following overnutrition (Willis et al. 2020) due to its energy-sensing activity; however, circulating levels of LECT2 may not be sensitive to acute exercise stimuli (Willis et al. 2019).

LECT2 is associated with metabolic stress and has been identified as a promoter of adhesion molecules by increasing the expression of ICAM-1 (Hwang et al. 2015). Also, as an enhancer of pro-inflammatory cytokines (de Oliveira Dos Santos et al. 2021), it contributes to the development of skeletal muscle insulin resistance in obesity (Yoo et al. 2017). However, very few studies have explored the clinical relevance of circulating LECT2 levels in humans (Yoo et al. 2017).

Furthermore, LECT2 enhances lipogenesis in 3 T3-L1 cells regardless of the impairment of fatty acid oxidation. Thus, LECT2 can be clinically useful for bodyweight management in obese individuals (Chikamoto et al. 2016) and an efficient, beneficial target for obesity-associated metabolic disorders, including IR, and may have a favorable impact on systemic low-grade chronic inflammation related to MetS (Jung et al. 2018b). Thus, LECT2 can be a critical biomarker to elucidated how hepatic steatosis is related to IR in obesity since its inhibition promotes insulin sensitivity in skeletal muscle.

Hepassocin, Also Called Hepatocyte-Derived Fibrinogen-Related Protein 1, HPS, and HFREP1

Hepatokines are secreted by the liver and influence insulin signaling in insulin-responsive tissues (Hung-Tsung et al. 2013). One of them, hepassocin (HPS), is a particular liver growth factor involved in regulating hepatocyte proliferation and liver regeneration (de Oliveira Dos Santos et al. 2021).

A significant association between HPS and fasting glucose, insulin resistance, and T2DM has been observed in human studies (Ou et al. 2015). However, causal inferences could not be made due to the cross-sectional design of the study.

Recently, it has been demonstrated that liver HPS levels are increased in patients with NAFLD (Hung-Tsung et al. 2013) and T2DM (Chang et al. 2014). Also, circulating HPS concentrations were found to be independently associated with FPG, HOMA-IR, pre-diabetes, and diabetes in humans by regulating mitogen-activated protein kinase ERK1/2 activity (Hung Tsung et al. 2016).

It has been proposed that HPS regulated hepatic lipid accumulation in HepG2 (human liver cancer cell line) cells, and overexpression of HPS participated in the development of NAFLD in mice, whereas deletion improves HFD-induced NAFLD (Hung-Tsung et al. 2013). In vivo and in vitro studies show that HPS contributes significantly to insulin resistance. In humans, serum levels of HPS are elevated in pre-diabetes, T2DM, and NAFLD because of their association with impaired fasting glucose, glucose intolerance, and insulin resistance (Hung Tsung et al. 2016).

Recent studies show that HPS is an obesity-induced hepatokine capable of promoting insulin resistance in skeletal muscle cells through epidermal growth factor receptor and c-Jun N-terminal kinase-mediated (EGFR/JNK) pathway. Therefore, HPS is proposed as a potential target for treating obesity-linked IR and T2DM (Jung et al. 2018b). Moreover, HPS can be a relevant biomarker for analyzing risk states for diabetes (de Oliveira Dos Santos et al. 2021).

Pigment Epithelium-Derived Factor

Pigment epithelium-derived factor (PEDF) is a secreted glycoprotein that possesses potent neuronal differentiating activity in human retinoblastoma cells. It has shown anti-angiogenic, anti-proliferative, neurotrophic, and immunomodulatory activities (Maeda et al. 2011). It is one of the most abundant proteins secreted by adipocytes; thus, impairing PEDF-adipose triglyceride lipase (ATGL) interaction can lead to IR, as seen in T2DM (Kuang-Tzu et al. 2018).

In vitro, PEDF directly increased the glucose uptake in hypoxic cardiomyocytes through the expression of glucose transporter 4 (GLUT4) and translocation on plasma membrane involving PI3K/AKT signaling (Yuan et al. 2019). Furthermore, in a mice model of obese T2DM with IR, PDEF has been shown to improve metabolic impairment through suppressing inflammatory and oxidative reactions in adipose tissue (Matsui et al. 2014). Additionally, PEDF has reduced 3 T3-L1 preadipocyte differentiation, limited adipogenesis, and ameliorated IR in diet-induced metabolic disorders in mice (Chin-Chuan et al. 2019).

Human studies have shown that PEDF is independently associated with fasting apolipoprotein B-48 (ApoB8) levels, implying that PEDF concentration may be a novel biomarker for postprandial hyperlipidemia in humans (Tahara et al. 2012).

Likewise, PEDF is positively related to muscle and fat tissue; both contribute to the circulating level of PEDF and potentially to its association with IR in children with obesity with or without T2DM (Tryggestad et al. 2015). Moreover, PEDF was 55% higher in T2DM children than normal-weight children but did not differ from obese children (Tahara et al. 2012); therefore, obesity would be more related to plasma PEDF than T2DM. Additionally, circulating levels of PEDF is increased in overweight young adults and is positively related to IR. Furthermore, recent pre-clinical and clinical studies have shown that skeletal muscle is also a source for circulating PEDF; therefore, contractions and exercise increase plasma PEDF (Sunderland et al. 2012).

Moreover, Tahara et al. propose 120-min post-load plasma glucose and PEDF values as markers and potential therapeutic targets of coronary artery inflammation in T2DM patients (Tahara et al. 2020). Therefore, PEDF seems like a possible therapy for obesity and cardiometabolic disorders.

Tsukushi (TSK)

Tsukushi (TSK) is an atypical small leucine-rich proteoglycan, newly identified as hepatokine, which increases circulating concentrations related to obesity (Wang et al. 2019). According to the analysis of secretome gene expression, TSK exerts powerful effects on adipose tissue thermogenesis and metabolic homeostasis (Xiong et al. 2019). In addition, it is related to the regulation of energy expenditure and is strongly associated with obesity, NAFLD, and NASH (de Oliveira Dos Santos et al. 2021).

Experimental studies show that inflammation and endoplasmic reticulum stress promotes hepatic TSK expression in mice, related to excessive hepatic fat accumulation (Mouchiroud et al. 2019b). Furthermore, a study by Wang et al. showed that inhibition of TSK increased sympathetic innervation and thermogenesis in brown adipose tissue, therefore protecting against diet-induced obesity in mice. However, Mouchiroud et al. could not show clear evidence that overexpression of TSK has any significant impact on the thermogenic capacity of brown adipose tissue, body weight gain, nor glucose homeostasis in mice (Mouchiroud et al. 2019a).

It has been shown that TSK reduces circulating high-density lipoprotein cholesterol, cholesterol efflux capacity, and the conversion of cholesterol to bile acid in the liver (Mouchiroud et al. 2019a). Additionally, TSK expression is increased in obesity, though it can be lowered after laparoscopic adjustable gastric banding; therefore, TSK expression is strongly related and is proposed to be the only significant predictor of TSK production (Grander et al. 2020).

Conclusions

Insulin resistance is the central physiopathological feature of metabolic syndrome and is closely related to NAFLD and T2DM. These chronic conditions are linked to obesity, particularly visceral adiposity, leading to an increased release of liver enzymes, known as hepatokines that are recognized and proposed as IR, NAFLD, and T2DM biomarkers. Some of these enzymes have positive activities related to reducing oxidative stress, lipogenesis, and improving insulin resistance and glucose homeostasis. However, some hepatokines are highly expressed in response to energy intake, obesity, NAFLD, and NASH. Therefore, the development of pharmacological inhibitors has been recommended as treatment or dual therapy in patients with IR and T2DM. In this group, DPP4i are the most studied hepatokine inhibitors, which are being found to be safe, tolerable, and cost-effective compared to traditional drug therapy alone in T2DM patients. On the other hand, since NAFLD is diagnosed

through ultrasound images, liver enzyme test, and IR does not show apparent diagnostic symptoms, the use of hepatokines as biological markers seems promising for early identification and treatment of these chronic conditions, especially in the early identification and treatment children, teenager, and young adults.

The research related to hepatokine identification and biological activity is still emerging; thus, elucidating the complex mechanisms behind its effects on chronic conditions is encouraging as treatment targets.

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Biological Markers of Insulin Sensitivity Links with Dietary Antioxidant

9

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Contents

Introduction 218
Applications to Diagnosis 218
Mini Dictionary of Terms 219
Key Facts of Insulin Sensitivity, Biomarkers, and Dietary Antioxidants 219
Summary Points 220
Markers of Liver Function and Insulin Resistance 220
Insulin Sensitivity 220
Adiponectin 221
Minerals in Insulin Resistance Sensitivity 226
Vitamins in Insulin Sensitivity 228
Conclusions 229
References 231

Abstract

Obesity plays an essential role in developing chronic conditions such as metabolic syndrome (MetS), nonalcoholic fatty liver disease (NAFLD), and T2DM, due to an increase in free radicals, insulin resistance, and disruption in beta cells activity. The increase of triglyceride storage in adipose tissue due to obesity results in adipocyte hypertrophy, in which infiltrated macrophages, enhancing the pro-inflammatory state. Both hepatokines and adipokines are involved in central and peripheral energy metabolism, immune response, insulin sensitivity, and lipid metabolism. Moreover, vitamins and minerals exert cytokines modulation activities, thus may be included as biomarkers, diagnose test for insulin resistance, and therapeutic targets for improving

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MetS, T2DM, and NAFLD. This chapter provides information on the relationship between essential micronutrient activity in insulin synthesis and secretion and its effects on obesity-related chronic conditions progression.

Keywords

Biological markers · Liver function · Insulin resistance · Vitamins · Minerals · vitamin D · vitamin K · Magnesium · Selenium · FGF21

Abbreviations

ANGPTL4	Angiopoietin-like protein 4
DPP4	Dipeptidyl peptidase – 4
Fetuin A/B	α2-Heremans-Schmid glycoprotein
FGF21	Fibroblast growth factor-21
GLP-1	Glucagon-like peptide-1
HMGB1	High mobility group box 1
PPAR-α/RXR	Peroxisome proliferator-activated receptor alpha/retinoic acid receptor
RBP4	Retinol-binding protein 4

Introduction

Insulin resistance plays a crucial role in the development of T2DM, metabolic disorders, and their comorbidities. Antioxidants such as vitamins and minerals exert important functions in regulating insulin sensitivity mediated by biomarkers related to liver gluconeogenesis, inflammatory responses, regulation of lipid productions, suppression of the appetite hormones, and other metabolic processes. Novel studies have shown the participation of minerals like calcium, magnesium, selenium, chromium, and zinc in the management of glucose metabolism disorders serving as regulators of insulin resistance, abdominal fat, oxidative stress, levels of myokines and cytokines; in a similar way, vitamins are related to performing regulation of chronic inflammation, a direct pathway to the development of insulin resistance, obesity, and T2DM. As an anti-inflammatory and antioxidant agent, vitamin C also improves insulin resistance; likewise, vitamin D can increase adiponectin levels and enhance insulin sensitivity. Moreover, vitamin E can reduce HbAc1, insulin, and malondialdehyde levels. Equally, vitamin k reduces proinflammatory interleukins secretion by improving insulin sensitivity; furthermore, B-complex vitamins, such as vitamin B6, B2, and B12 supplementation, can improve glycemic control and insulin resistance.

Applications to Diagnosis

In this chapter studies related to biological markers of insulin sensitivity links with dietary antioxidant and minerals has been reviewed, which show promising use of antioxidants such vitamins and minerals and its relation to the following biomarkers

linked to insulin sensitivity such as adiponectin, adropin, ANGPTL4, FGF21, HMGB1, GLP-1, interleukins, RBP4, irisin, fetuin A/B, and DPP4 levels like an alternative to diagnose insulin resistance and related metabolic disorders. Moreover, micronutrients such as vitamins and minerals may improve insulin sensitivity by suppressing hepatic glucose production and inflammatory responses allowance the development of micronutrients therapies to optimize insulin sensitivity and prevent type 2 diabetes progression.

Mini Dictionary of Terms

- **Adiponectin**: A protein hormone and adipokine secreted by adipocytes with the highest plasma concentration, modulated lipid metabolism, and glucose levels, improving insulin sensitivity by its antioxidant and anti-inflammatory effects.
- **Adropin**: A protein that exerts a regulatory function of energy homeostasis and insulin response suppressing hepatic glucose production.
- **ANGPTL4**: A glycoprotein highly expressed in liver and adipose tissue, is the target of PPARs and plays the physiological role in lipid metabolism, inhibits the LPL activity allowing for the enhanced circulation of TG.
- **DPP4**: An enzyme responsible for deleting incretins like GLP-1 and DPP4 inhibitors, plays a significant role in glucose metabolism, improving IR.
- **Fetuin A/B**: Glycoproteins, which can bind to the insulin receptor and inhibit insulin signaling; increased levels are related to insulin resistance.
- **FGF21**: A hepatokine, which expression increase by the PPAR-γ/RXR heterodimer activation, stimulated the glucose uptake in adipocytes, and regulated the sugar intake by its FGF21 receptors in the hypothalamus.
- **HMGB1**: A protein secreted by immune cells. As a cytokine mediator of inflammation in most cases, the interaction with TLRs like TLR4 results in the upregulation of NF-κB, increasing the production and release of cytokines.
- **IL-32**: A novel pro-inflammatory cytokine produced by T lymphocytes, natural killer cells, epithelial cells, and blood monocytes. Induce aggravation of inflammation.
- **Irisin**: A hormone that regulates fat tissue and blood metabolism by exercise, converting white adipose tissue to brown adipose tissue, improving IS.
- **Myostatin**: Myokine, produced by myocytes, inhibiting muscle cell growth, loss of action of this myokine is related to improving IS.
- **RBP4**: An adipokine whose principal role is to transport retinol from the liver to the peripheral tissues; increased adipokine levels are related to IR.

Key Facts of Insulin Sensitivity, Biomarkers, and Dietary Antioxidants

1. Plasma concentration of adiponectin can be considered an important monitor index for obesity, due to its role in fat mass regulation and fat browning markers expression.

2. Angiopoietin-like 4 (ANGPTL4) plays a major role in triglycerides metabolism through suppressing pancreatic and lipoprotein lipases.
3. FGF21 levels are increased in chronic conditions such as MetS, obesity, dyslipidemia, insulin resistance, T2DM, NAFLD, and coronary artery disease.
4. Low serum level of chromium has been commonly found in T2DM patients.
5. Among T2DM patients, there is a 25–39% prevalence of deficit in magnesium.

Summary Points

- Circulating IL-6, IL-36, IL-29, and irisin may represent possible therapeutic targets for insulin resistance as having been found to increase in obese and MetS patients and biomarkers for diagnosis or treatment of T2DM.
- As a novel adipokine, zinc-α2-glycoprotein (ZAG) plays an important role in modulating insulin sensitivity and have been shown to reduce insulin resistance.
- Clinical studies have shown that obese patients have lower serum adropin, and it is inversely related to fasting glucose, insulin, and anthropometric measures.
- Vitamin D deficiency may be involved in the pathogenesis of metabolic disturbances such as MetS and polycystic ovary syndrome and increase the risk of developing T2DM.
- Vitamin K2 inhibits insulin resistance by enhancing mitochondrial activity through SIRT1 signaling, also higher phylloquinone (vitamin K1) levels are related to a reduced risk of T2DM, turning vitamin K1 and K2 into essential nutrients for human health.

Markers of Liver Function and Insulin Resistance

Insulin Sensitivity

Diabetes mellitus is a long-term metabolic disruption characterized by hyperglycemia resulting from disorders in pancreatic beta-cell function, insulin secretion, or activity (Qu et al. 2018). Therefore, type 2 diabetes mellitus (T2DM) results from a hormonal imbalance, where insulin and glucagon cannot regulate and stabilize blood glucose levels and limit the release of adipose fatty acids (Alfatlawi et al. 2021).

Moreover, obesity plays a vital role in the development of chronic conditions such as metabolic syndrome (MetS), nonalcoholic fatty liver disease (NAFLD), and T2DM, due to an increase in free radicals, insulin resistance (IR), and disruption on beta cells activity (Safarpour et al. 2020). The increase in adipose tissue due to obesity results in adipocyte hypertrophy, in which macrophages infiltrates, enhancing proinflammatory cytokines.

Adipose tissue, as an endocrine organ, is responsible for adiponectin and leptin secretion. However, during obesity, these hormones failed to regulate glucose metabolism and insulin sensitivity. Therefore, insulin resistance alters glucose uptake in insulin-sensitive tissues, primarily skeletal muscle. Additionally, IR is

the primary mechanism in developing MetS and T2DM, and is positively correlated with weight gain, primarily visceral fat accumulation. It is known from epidemiological, preclinical, and clinical studies that weight loss promotes insulin sensitivity in obese patients as a result of a reduction in IR (Das and Choudhuri 2020), which leads to metabolic and hemodynamic homeostasis known as counteracting MetS (Liu et al. 2020).

Adiponectin

Adiponectin Is the most abundant cytokine (Choubey et al. 2020), present as full-length adiponectin and as a C-terminal globular fragment exclusively secreted by the adipose tissue known as an anti-inflammatory adipokine (Khoramipour et al. 2021). Also, adiponectin plays a significant role in central and peripheral energy metabolism and is also involved in lipid metabolism, immune response, and insulin sensitivity (Jo et al. 2021).

Moreover, adiponectin displays important antiatherogenic and antihyperglycemic activities by reducing gluconeogenesis and increasing insulin sensitivity (Moon et al. 2019). In contrast to leptin, plasma adiponectin levels are reduced in patients with insulin resistance, obesity, coronary artery disease, and T2DM, particularly those with visceral adiposity (Gariballa et al. 2019). Therefore, it is related to β-cell dysfunction and has shown to have different antihyperglycemic activity according to ethnicity, being better in Caucasians (Hakim et al. 2021).

Globular adiponectin therapy reduces serum glucose, triglycerides, insulin, reverses insulin resistance, and decreases fat accumulation in the liver and muscle, which could be attributed to enhanced glucose uptake in muscle and adipose tissues (Li et al. 2020). Therefore, adiponectin is proposed as a potential antidiabetic, anti-inflammatory, and antiatherogenic therapeutic tool, for obesity and related comorbidities such as T2DM and cardiovascular disease (Gariballa et al. 2019).

Moreover, T2DM patients with high plasma adiponectin showed better antioxidant capacity, the large size of high-density lipoprotein (HDL), and a decreased apolipoprotein C3, a potent inhibitor of lipoprotein lipase (Dias et al. 2021). Several studies indicate that leptin-to-adiponectin ratio (LAR) could be a very acute biomarker of atherosclerosis progression of insulin resistance (Piumngam et al. 2021).

Adiponectin plasma concentrations can be considered an essential monitor index for obesity due to its role in fat mass regulation and fat browning markers expression. Also, AdipoR1,AdipoR2, and T-cadherin receptors have an intermediate affinity for globular and full-length molecular weight adiponectin forms in the liver, thus controlling energy metabolism inflammatory responses, and insulin sensitivity (Peng et al. 2018). Furthermore, its receptors have been shown to improve glucose regulation and enhance fatty acid oxidation, making adiponectin an important target for obesity treatment (Bais and Patel 2019) and an early diagnostic marker for insulin resistance along with anthropometric evaluation. Furthermore, adiponectin has been proposed as a promising biomarker for the T2DM treatment and amelioration of disease development (Biercewicz et al. 2020).

Adropin, Angiopoietin-lLike Protein 4 (ANGPTL4)

Adropin is a peptide-structured hormone with 76 amino acid precursors involved in lipid cardiovascular system functions and glucose metabolism, thus related to insulin resistance and adipose accumulation (Zarrati et al. 2019). In addition, clinical studies have shown that obese patients present reduced serum adropin, and it is inversely related to glucose, insulin, and body composition (Erman et al. 2021).

Adropin is mainly expressed in the liver and brain; although it has been identified in other tissues such as the lung, muscles, heart and gastrointestinal tract, it is encoded by the energy homeostasis-associated gene (Enho) (Yang et al. 2020). A mice model of liver steatohepatitis has shown that adropin is influenced by exercise, leading to a rise in serum levels and a suppression of NLRP3 inflammasome activation and protein-complex related to inflammatory conditions. Also, it has been shown to reduce pro-inflammatory cytokines IL-1β, IL-6, and TNFα steatohepatitis (Yang et al. 2021).

Additionally, preclinical studies have observed that adropin can improve glucose homeostasis, lipid disorders in obesity, reduce the expression of pyruvate dehydrogenase kinase 4 (PDK4), a gene involved in T2DM development, inhibits the production of hepatic glucose, therefore enhancing glucose uptake and insulin sensitivity (Luo et al. 2020), and also can protect against hepatocytes injury (Jasaszwili et al. 2020). Adropin levels are downregulated in diverse cardiovascular diseases, and lower serum adropin in T2DM patients is considered a risk factor for endothelial dysfunction (Zhang and Li 2019).

Angiopoietin-like 4 (ANGPTL4) is mainly expressed in liver and adipose tissue, playing a major role in triglycerides metabolism through suppressing pancreatic and lipoprotein lipases (Janssen et al. 2018). Furthermore, clinical studies have shown that patient carriers of E40K-inhibiting mutation in ANGPTL4 present lower plasma triglyceride levels and have a reduced risk of coronary artery disease compared to noncarriers (Abid et al. 2016).

Moreover, evidence suggests that inhibition of ANGPTL4 improves glucose tolerance and insulin resistance through gut microbiota modulation, reducing triglycerides levels and cardiovascular disease risk (Janssen et al. 2018). ANGPTL4 can be regulated by diet through an increased concentration of nonesterified fatty acids (NEFA)in the bloodstream. Additionally, preclinical studies have shown that expression of ANGPTL4 is upregulated by fatty acids exposures, such as palmitic acid, oleic acid and arachidonic acid in cell studies (Nielsen et al. 2015). Therefore, ANGPTL4 is considered a possible target for cardiometabolic disease management and is a downstream target gene of peroxisome proliferator-activated receptors (PPAR), extensively used in T2DM lipid disorders due to its capability to trigger adipocyte lipolysis (Wang et al. 2016).

DPP4, GLP-1

Adipose tissue is identified as an endocrine organ due to its ability to release metabolites and bioactive factors, known as adipokines. One of them is dipeptidyl peptidase-4 (DPP4), an adipokine related to central adiposity and insulin resistance (Siciliano et al. 2019). DPP4 is a transmembrane glycoprotein that is expressed in the liver, kidney, gut, lung, and endothelial cells. Moreover, both its circulating form and

membrane-bound activities are related to chronic inflammation, MetS, and T2DM (Romacho et al. 2020).

DPP4 limits insulin-induced protein kinase B (AKT) phosphorylation in skeletal and adipose tissue (Siciliano et al. 2019). Insulin resistance markers, such as serum insulin and HOMA-IR, are positively correlated with DPP4 expression in visceral adipose tissue; furthermore, DPP4 levels are increased in obesity and T2DM (Hong et al. 2017).

Additionally, DPP4 can hydrolyse glucagon-Like peptide-1 (GLP-1), a hormone that stimulates insulin release, reduces energy intake, and slows gastric emptying (Rotella et al. 2005). Therefore, dipeptidyl peptidase 4 inhibitors (DPP4i) have been developed and are widely used as incretin-based therapies for T2DM, such as gemigliptin, a strong DPP4i is approved and applied for T2DM treatment in over ten countries around the globe (Dutta et al. 2021). DPP4i extends the half-life of incretins, such as GLP-1, and have been shown to provide glycemic control (Pérez-Durillo et al. 2018). Moreover, DPP4i and GLP-1 receptor agonist (GLP-1 Ra) can enhance insulin secretion and are extensively used to treat T2DM; they can also reduce the rate of developing postpartum diabetes (Chen et al. 2020).

Glucagon-like peptide-1 GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are the principal stimuli of postprandial glycemia in response to enteral glucose. Both are secreted by L cells of the intestinal through the post-translational processing of proglucagon (Lin et al. 2021). Therefore, GLP1-Ra, which can resist DPP4 hydrolysis and prolongs the activation of GLP1 receptor, which can enhance endogenous insulin response and limit glucagon secretion, are often the first injectable treatment recommended in T2DM before insulin therapy (Norrbacka et al. 2021).

GLP-1Ra can be divided into short-acting, exenatide and lixisenatide, and long-acting, like albiglutide, dulaglutide, liraglutide, and semaglutide (Onoviran et al. 2019). Lixisenatide has been shown to significantly reduce glycated hemoglobin (HbA1c) postprandial glycemic levels in T2DM (Liu et al. 2021); together with sodium-glucose cotransporter 2 inhibitor, it has shown to enhance weight loss, control lipids, and blood pressure (Castellana et al. 2019).

Fetuin-A and Fetuin-B

Fetuin-A and fetuin-B belong to the fetuin family and are known as hepatokine that share around 22% similarity in their sequence (Ebert et al. 2017). Fetuin-A, also described as α2-Heremans-Schmid glycoprotein (α2-HS-glycoprotein), is a multifunctional glycoprotein produced mainly by the liver and adipose tissue; it has a place in the 3q27 gene in the human genome, related to MetS and T2DM (Ghadimi et al. 2021). Fetuin-A is involved in a diverse range of physiological conditions and a metabolic regulator that induces metabolic dysfunction (Stefan et al. 2008). Also, it has been linked to insulin resistance due to inhibiting insulin receptors of tyrosine kinase activity, insulin signaling (Fatima et al. 2020), and promoting inflammation in immune cells and adipocytes. Therefore, it may be involved in the development of MetS (Zhang et al. 2020). However, modest and significant weight loss has reduced serum fetuin-A and improved insulin sensitivity (Ren et al. 2021).

On the other hand, fetuin-B, a novel hepatokine (El-Ashmawy and Ahmed 2019), is significantly increased in NAFLD and related to insulin resistance and glucose intolerance. Additionally, clinical studies have shown an increased plasma fetuin-B in obese patients, gestational diabetes mellitus (GDM) (Kralisch et al. 2017), and in women with polycystic ovary syndrome (PCOS), and T2DM patients (Li et al. 2018). However, GLP-1Ra therapy can significantly reduce fetuin B levels in patients with PCOS (Mokou et al. 2020). Thus, plasma fetuin-B may be considered an autonomous predictor and biomarker of insulin resistance (Qu et al. 2018).

FGF21

Fibroblast growth factor 21 (FGF21), formed by 209 amino acids, has been described as a pleiotropic hormone. It is primarily secreted in the liver and adipose tissue, promoting fatty acid oxidation, glucose metabolism, and insulin sensitivity (Urraza-Robledo et al. 2021). Also, preclinical studies have shown that FGF21 prevents high fat-induced obesity, stimulates glucose uptake, and decreases lipogenesis, which would be mediated through modulation of adiponectin (Ángel et al. 2021). Also, its attenuation may worsen glucose impairment during the transition from prediabetes to diabetes (Kondeti et al. 2021)

FGF21 has been proposed as an effective biomarker for NAFLD (Tas et al. 2020).

Epidemiological studies have shown increased FGF21 levels in chronic conditions such as MetS, obesity, dyslipidemia, insulin resistance in T2DM, NAFLD, and coronary artery disease (Magdas et al. 2019). Nonetheless, a very low level of circulating FGF21 is linked to diabetic retinopathy development (Jung et al. 2017).

FGF21 is the first known endocrine signal that is activated by protein restriction rather than energy deprivation. It is involved in the regulation of β-oxidation, ketogenesis, neoglucogenesis, and lipogenesis via activating transcription factor 4 (ATF4)(Chalvon-Demersay et al. 2019) and actively elevated in low-protein environments, also in fasting, overfeeding, ketogenic, and high-carbohydrate diets (Laeger et al. 2017). Additionally, physical activity may be an essential therapy for increasing and recovering sensitivity to FGF21 (da Silveira Campos et al. 2017). FGF21 have been shown to increase energy expenditure through enhancing browning in subcutaneous white adipose tissue and may increase preservation of muscle mass and resting metabolic rate (Kravchychyn et al. 2020).

HMBG1, Myostatin

High mobility group box 1 protein (HMGB1) is a highly conserved nuclear protein that is widely released from activated monocytes and macrophages after pro-inflammatory stimuli and injured or dying cells, which aggravates inflammatory processes and activates inflammatory responses by stimulating toll-like receptor 4 (TLR4) (Yi et al. 2020).

Moreover, HMGB1 can be an independent risk factor for T2DM and served to predict the occurrence of T2DM combined with chronic obstructive pulmonary disease (Huang et al. 2019). Additionally, animal studies have shown that neutralization of HMGB1 can significantly improve liver damage and survival in a model of acute liver failure (Tian et al. 2020). Moreover, HMGB1 can be used as an

immunomodulatory factor due to its effects on modulation of DNA recombination, replication, repair, and transcription (Zhang et al. 2018). Also, it has been observed that dietary antioxidants, such as vitamins, minerals, and phytochemicals, can be used as possible therapies for acute inflammatory process induced by oxidative stress induced possibly through an HMGB1-mediated pathway, as has been seen in patients receiving mechanical ventilation (Patel et al. 2020).

Myostatin (GDF-8) is a member of the transforming growth factor-beta (TGF-β) superfamily of secreted growth and differentiation factors (Ma et al. 2016). It is mainly secreted in skeletal muscle and can also be detected in other tissues, such as fat, liver, and kidney. The principal activity of myostatin is to regulate skeletal muscle growth and development negatively. However, according to the situation, it can also exert a dual function, either inhibiting or promoting adipogenesis (Deng et al. 2017).

Evidence suggests that myostatin suppression by mutation can increase insulin sensitivity; therefore, myostatin inhibition has been proposed as a therapeutic approach in T2DM (Yan et al. 2019). Nonetheless, the exact mechanism of myostatin inhibition in improving muscle glucose uptake is still unclear (Eilers et al. 2020).

Interleukins, Irisin, RBP4

Among the cytokines related to MetS and T2DM, interleukin 6 (IL-6) is the most highlighted, mainly due to its positive correlation with obesity, primarily abdominal adiposity, which can influence oxidation state and modify inflammation markers (Drehmer et al. 2020). Therefore, IL-6 and IL-10 genetic variations are being proposed as biomarkers for early screening and diagnosis of T2DM (Ayelign et al. 2021).

Concerning other interleukins, clinical studies have shown that Interleukin-29 (IL-29), a type 3 interferon family member, is significantly higher in obese patients, and its inhibition can decrease inflammatory cytokine production in macrophage-adipocyte coculture systems (Lin et al. 2020). Furthermore, studies have shown an increase in IL- 36α and IL-36γ and reduced IL-36Ra expression in T2DM patients, directly proportional to inflammation and blood lipid levels (Li et al. 2021). In addition, there is a decline in serum levels of IL-17/IL-22 in the early onset of metabolic diseases, which is strongly related to a modification in the gut microbiome (Zhou et al. 2020).

On the other hand, Irisin, a myokine that is expressed and secreted principally by muscle tissue, regulates energy metabolism through thermogenesis activation (Miazgowski et al. 2020). It is released due to peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC-1α) stimuli. Irisin has been shown to improve fatty acid oxidation and glucose metabolism, therefore diminishing atherosclerosis progression (Saiel et al. 2021).

Irisin has positive effects on the brain, bone, and adipocytes; it enhances thermogenesis and may increase brown adipose tissue over white fat. It is also involved in physical activity benefits (Kim et al. 2018). Moreover, human studies have shown that T2DM and GDM patients present lower irisin and PGC-1α (Al-Ghazali et al.

2020). Therefore circulating IL-6, IL-36, IL-29 may represent possible therapeutic targets for insulin resistance.

Secondly, retinol-binding protein-4 (RBP4) is a specific carrier for vitamin A (retinol) in the blood that has been newly identified as hepatokine. However, RBP4 is also secreted by adipocytes, and it may be involved in the development of insulin resistance (Liu et al. 2019). Serum RBP4 levels are significantly increased in T2DM, obese individuals, and a high risk of cardiovascular disease (Zhou et al. 2018). Moreover, high levels of RBP4-to-vitamin A ratio have been found to be related to diabetic retinopathy severity (Rostamkhani et al. 2020).

Minerals in Insulin Resistance Sensitivity

Calcium, Magnesium

Evidence suggests that trace minerals are involved in the physiopathology of obesity, insulin resistance, and other obesity-related chronic conditions. Moreover, a relationship between calcium deficiencies and zinc has been observed in overweight and obese patients (Wu et al. 2019). Moreover, total serum and ionized calcium (Ca) and magnesium (Mg) contents and the Ca/Mg ratio have been found to be lower in T2DM patients (Moustafa 2016).

A recent animal study has shown that calcium exerts positive effects even in a high-fat diet (HFD) environment, reducing the risk of insulin resistance by improving the hepatic and muscle insulin sensitivity by restoring adipokine, particularly adiponectin secretion; meanwhile, a calcium-deficient HFD results in an increased progression to insulin resistance (Das and Choudhuri 2020).

Oral calcium supplementation improves insulin sensitivity in patients with T2DM and hypertension. Moreover, supplementation with vitamin D and calcium have improved insulin sensitivity (Gagnon et al. 2014). Furthermore, clinical studies in healthy individuals have shown that calcium fructoborate supplementation can improve biomarkers of cardiovascular risk, such as lipid profile and inflammatory cytokines (Rogoveanu et al. 2015). Additionally, a recent meta-analysis has shown that high doses of vitamin D and calcium co-supplementation for less than 4 months can significantly reduce fasting glucose, insulin, thus improving insulin resistance (Asbaghi et al. 2019).

On the other hand, magnesium, an essential trace element, takes part in various physiological processes; its ion form protects vascular endothelial cells from oxidative stress by acting as a calcium antagonist. Moreover, magnesium deficiency leads to increased insulin resistance and metabolic disorders (Feng et al. 2020) that augments the risk of T2DM development and progression. Among T2DM patients, there is a 25–39% prevalence of deficit in magnesium (Liu et al. 2020).

Epidemiological studies show that magnesium intake is inversely associated with insulin resistance (Suliburska et al. 2014). Moreover, circulating magnesium levels are significantly lower in T2DM patients than nondiabetic patients, thus magnesium deficiency can be used as a prognostic factor for T2DM progression and

complications (Patil et al. 2020). Therefore, magnesium monitoring and supplementation should be considered in T2DM management since it can significantly improve the HOMA-IR index and fasting glucose in diabetic and nondiabetic subjects (Simental-Mendía et al. 2016). This effect may be associated with its anti-inflammatory effect on HMGB1/TLR4/ and nuclear factor-kappa B (NF-κB) signal pathway (Jiang et al. 2020).

Chromiun, Selenium, Zinc

Chromium trivalent complex has been shown to improve insulin sensibility significantly and exerts moderate lowering lipids activity (Król et al. 2012). Therefore, low serum level of chromium has been commonly found in T2DM patients (Hajra et al. 2016). Furthermore, preclinical studies have shown that chromium malate positively influences glucose levels and insulin resistance by regulating proteins production and genes expression in glucose uptake and insulin sensitivity signaling pathways (Feng et al. 2018). Moreover, biotin and chromium co-supplementation have considerable benefits over glucose transportation, PPAR- γ, and insulin receptor substrate 1 (IRS- 1) in a HFD model (Orhan et al. 2019).

Human studies have shown that chromium supplementation can significantly reduce high-sensitivity C-reactive protein (hs-CRP), plasma malondialdehyde (MDA) levels, blood pressure, proinflammatory cytokines, and increase significantly total antioxidant capacity (Zhang et al. 2021). In addition, chromium picolinate has been shown to significantly reduce triglycerides, HOMA-IR, and Fetuin-A and improve insulin sensitivity in patients with NAFLD (Moradi et al. 2021).

Secondly, selenium is an essential micronutrient that integrates into proteins to form 25 selenoproteins. It is present in a wide range of foods, such as Brazil nut, fish, liver, and chicken, Its antioxidant activity has been widely recognized (Saxena et al. 2017). Additionally, selenium has insulin-mimetic activity and favors insulin synthesis; however, its effects on insulin sensitivity are dose and time-dependent (Fontenelle et al. 2018). Moreover, serum Se levels have a negative correlation with IL-6 and growth/differentiation factor-15 (GDF-15), which regulates inflammatory pathways; in addition, selenium deficiency has been found to be related to NAFLD development and progression (Prystupa et al. 2017).

On the other hand, zinc is also an essential micronutrient for human health; it is present in over 300 metalloenzymes and 2500 translation factors and is involved in diverse cellular functions and metabolic pathways (King et al. 2015). Moreover, zinc has been proposed as a supplement for modulating adipokines secretion due to its endocrine roles and is essential for insulin synthesis (Tabatabaie et al. 2021).

As a novel adipokine, zinc-α2-glycoprotein (ZAG) plays an important role in modulating insulin sensitivity (Balaz et al. 2014) and have been shown to reduce insulin resistance, thus preventing and improving T2DM. Furthermore, circulating ZAG levels are reduced in patients with MetS. Thus, they have been suggested as an effective biomarker for the MetS diagnosis (Lei et al. 2017). Also, human studies have shown that zinc supplementation can restore adiponectin levels and increase circulating HDL in T2DM patients (Asghari et al. 2019).

Vitamins in Insulin Sensitivity

Vitamin C, E

Evidence suggests vitamin C inhibits inflammation, modulating TNF-α, mitogen-activated protein kinase (MAPKs), NF-κB signaling; moreover, vitamin C deficiency leads to the aggravation of liver fat accretion (Qing et al. 2018). Furthermore, clinical studies have shown that patients with insulin resistance present lower plasma vitamin C levels (Donin et al. 2016), and oral intake of vitamin 1500 mg/day C for 3 months can be effective for reducing obesity-related comorbidities through enhancing insulin sensitivity and reverting obesity (Ghanwat and Sontakke 2019). Moreover, vitamin C supplementation has been shown to significantly reduce triglycerides, insulin concentration, and insulin resistance, which resulted in a decreased blood glucose and HbA1c in T2DM patients (Sanguanwong et al. 2016).

On the other hand, α-tocopherol (vitamin E) had beneficial effects on lipid profile and anthropometric measurements (Dass et al. 2018). Moreover, low vitamin E concentrations are robustly related to insulin resistance, low β-cell function, and high receptor for advanced glycation end products (RAGE) levels, which is considered an independent risk factor for atherosclerosis in T2DM (Chua et al. 2020).

Vitamin E supplementation significantly reduces HbA1c and fasting insulin concentrations in subjects with low serum vitamin E concentrations and poor glycemic control and also a significant reduction in the troponin T levels and cardiovascular risk measure through Framingham Risk Score (Praveen et al. 2019), and may exert beneficial effects reducing diabetic retinopathy progression (Chatziralli et al. 2017). Furthermore, physical activity significantly increased the modulatory effects of vitamin E on dyslipidemia, insulin resistance, blood pressure, oxidative stress, inflammation, leptin, and adiponectin in animal studies (Dallak et al. 2020).

Vitamin D, K, B

Calciferol (vitamin D) has been recognized for its relevant nonskeletal functions, primarily endocrine activities like significantly increased adiponectin concentration in GDM mothers after delivery (Hosseinzadeh et al. 2020). Likewise, human studies have shown that higher vitamin D levels are related to better postprandial glucose oxidation and enhanced insulin sensitivity (Pathak et al. 2017).

It is estimated that around 1 billion people around the globe present different degrees of vitamin D deficiency (Safarpour et al. 2020); moreover, vitamin D deficiency may be involved in the pathogenesis of metabolic disturbances such as MetS (Buchmann et al. 2021), PCOS, and increase the risk of developing

T2DM (Pramono et al. 2020). Moreover, vitamin D has been independently related to insulin resistance in T2DM (Zhao et al. 2021). Therefore, vitamin D supplementation has been shown to significantly increase insulin sensitivity and β-cell function, particularly in prediabetic patients (Munibuddin et al. 2020).

Vitamin K2 has been shown to improve insulin sensitivity and glucose metabolism through modulation of adipokine and anti-inflammatory and lipid-lowering activities related to its receptor vitamin K-dependent protein (osteocalcin) (Manna and Kalita 2016). Moreover, natural sources of vitamin K2 prevents insulin resistance by enhancing mitochondrial function through sirtuin 1 (SIRT1) signaling (Su et al. 2021). Also, higher phylloquinone (vitamin K1) levels are related to a reduced risk of T2DM, turning vitamin K1 and K2 into important nutrients for human health (Zwakenberg et al. 2019).

Secondly, vitamin B12 deficiency and borderline levels have been strongly associated with the dose of metformin (Al Saeed and Baraja 2021), whereas B12 supplementation improves glycemic control and insulin resistance in T2DM patients (Satapathy et al. 2020).

Therefore, the inclusion of B12 testing and magnesium in metformin-treated patients, particularly those with morbid obesity, is recommended (Wåhlén et al. 2017).

Between B-complex vitamins, pyridoxine (Vitamin B6) supplementation has been shown to enhance glucose tolerance, insulin secretion, reduce hepatic fat accretion, and prevent endothelial dysfunction. Furthermore, patients with fatty liver showed reduced vitamin B6 levels; thus, vitamin B6 supplementation may be adequate to prevent MetS (Liu et al. 2016). In addition, riboflavin (vitamin B2) deficiency leads to an increase in pro-inflammatory activity of adipocytes, which contributes to worsening of chronic inflammation, which is strongly related to obesity (Mazur-Bialy and Pocheć 2017).

Conclusions

Insulin resistance is the major leading cause of obesity, metabolic syndrome, NAFLD, and T2DM. Recently, the activity of hepatokines and adipokines have been highlighted due to their insulin modulation action. Moreover, trace minerals and vitamins are involved in obesity-related metabolic conditions since low serum levels of these micronutrients are strongly related to insulin resistance and T2DM progression. In addition, supplementation therapy has been positive in patients with metabolic disorders and may modulate hepatokines and adipokines activities (Figs. 1 and 2).

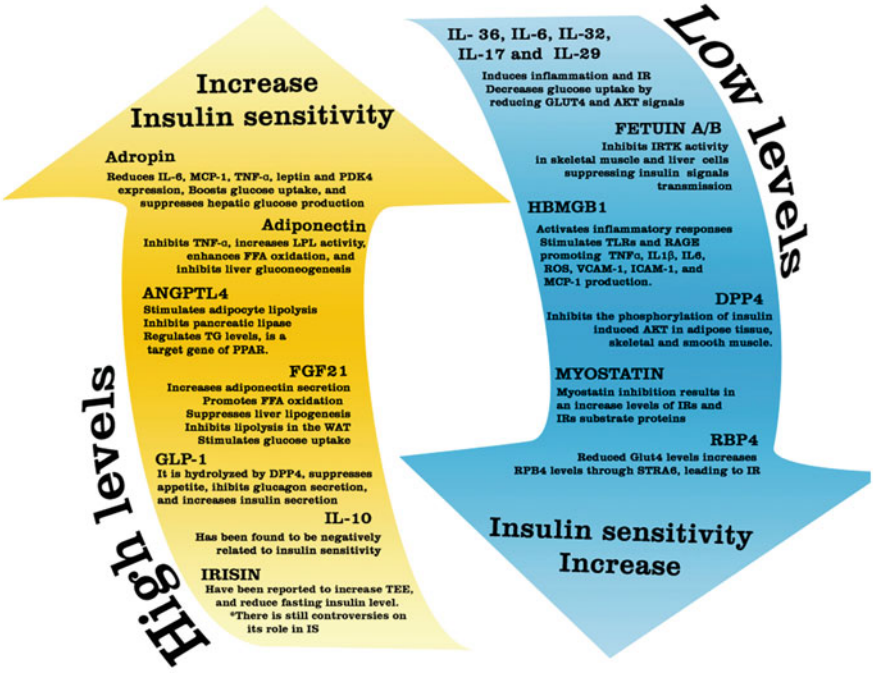

Fig. 1 Biomarker's effects related to insulin sensitivity. Higher levels in circulating adropin, adiponectin, ANGPTL4, FGF21, GLP-1, IL-10, and irisin leads to an increase in insulin sensitivity levels. On the other side, lower IL-36, IL-6, IL-32, IL-17, IL-29, fetuin A/B, HBMGB1, DPP4, myostatin, and RBP4 increases insulin sensitivity levels. IL-6, Interleukin-6; MCP-1, Monocyte Chemoattractant Protein 1; TNFα, Tumor Necrosis Factor-α; PDK4, Pyruvate dehydrogenase kinase 4; LPL, Lipoprotein lipase; FFA, Free fatty acid; TG, Triglyceride; PPAR, Peroxisome proliferator-activated receptors; WAT, White adipose tissue; IL-10, Interleukin-10; TEE, Total energy expenditure; IS, Insulin sensitivity; IL-36, Interleukin-36; IL-32, Interleukin-32; IL-17, Interleukin-17; IL-29, Interleukin-29; GLUT4, Glucose transporter 4; AKT, Protein kinase B; IRTK, Insulin receptor tyrosine kinase; TLRs, Toll-Like Receptors; RAGE, Receptor for advanced glycation end products; IL-1β, Interleukin-1β; ROS, Reactive oxygen species; VCAM-1, Vascular Cell Adhesion Molecule 1; ICAM-1, Intercellular Adhesion Molecule 1; MCP-1, Monocyte Chemoattractant Protein 1; IRs, Insulin receptors; STRA6, Stimulated by retinoic acid 6; IR, Insulin resistance

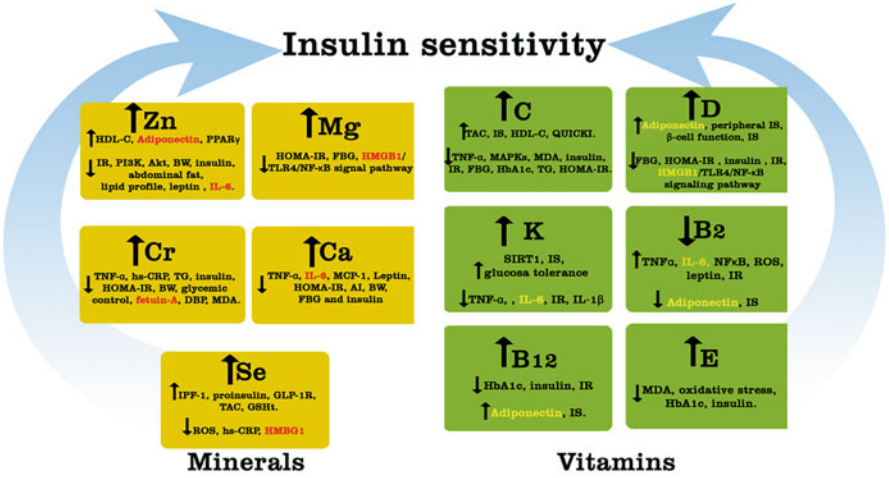

Fig. 2 Vitamins and minerals effects in insulin sensitivity and biological biomarkers. Human and animal studies included in this chapter are presented in this graphic, which shows that high levels of vitamins and minerals enhance insulin sensitivity; nevertheless, low levels of vitamin B2 are associated with decreasing insulin sensitivity. HDL-C, High-density lipoprotein cholesterol; PPARγ, peroxisome proliferator activated receptor gamma; IR, Insulin resistance; PI3K, Phosphoinositide 3-kinase; AKT, Protein kinase B; BW, Body Weight; IL-6, Interleukin-6; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; FBG, Fasting blood glucose; TLR4, Toll like receptor 4; NFkB, Nuclear factor kappa-light-chain-enhancer of activated B cells; TNFα, Tumor Necrosis Factor-α; hs-CRP, high-sensitivity C-reactive protein; TG, Triglycerides; DBP, Diastolic blood pressure; MDA, Malondialdehyde; MCP-1, Monocyte Chemoattractant Protein 1; AI, Adiposity index; IPF-1, Insulin promoter factor-1; GLP-1R, Glucagon-like peptide-1 receptor; TAC, total antioxidant capacity; GSHt, Total glutathione; ROS, Reactive oxygen species; IS, Insulin sensitivity; QUICKI, Quantitative Insulin Sensitivity Check Index; MAPKs, Mitogen-activated protein kinase; HbA1c, Glycosylated hemoglobin; SIRT1, Sirtuin-1; IL-1β, Interleukin-1β; B12, Vitamin B12; B6, Vitamin B6

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Serum Uric Acid and Metabolic Markers in Diabetes

10

eGFR, HbA1c, and Beyond

Mohamed Rafiullah and Khalid Siddiqui

Contents

Introduction 240
Metabolic Markers in Diabetes 241
Uric Acid 242
Uric Acid in Diabetes Pathophysiology 243
Uric Acid as a Biomarker in Diabetes 245
Serum Uric Acid as a Predictor of Diabetes Risk 245
Serum Uric Acid, Blood Glucose Level, and HbA1c 249
Uric Acid in Diabetic Complications 249
Uric Acid in Macrovascular Complications 250
Uric Acid in Microvascular Complications 251
eGFR and Serum Uric Acid Levels 252
Applications to Prognosis and Other Diseases or Conditions 253
Applications to Prognosis 253
Applications to Other Diseases or Conditions 253
Mini-Dictionary of Terms 254
Key Facts of Serum Uric Acid and Metabolic Markers in Diabetes: eGFR, HbA1c, and Beyond 254
Key Facts of Uric Acid and Diabetes 254
Key Facts of Uric Acid and Diabetic Complications 254
Summary Points 255
References 255

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Abstract

Uric acid, an organic compound, is the end product of purine metabolism in the liver. Hyperuricemic conditions are implicated in the pathogenesis of diabetes. Elevated uric acid level causes oxidative stress, leads to the activation of inflammatory processes, inhibits insulin pathway, and increases hepatic glucose production and dysfunction and death of pancreatic beta cells. Serum uric acid levels are found to be high in the early stages of impaired glucose metabolism. The role of uric acid in the development of abnormal glucose metabolism by causing insulin resistance, impaired insulin secretion, and beta-cell dysfunction suggests it could be an early indicator of diabetes risk. Uric acid is involved in inducing oxidative stress and endothelial dysfunction and also worsens insulin resistance. All these factors are involved in the development of diabetic complications as well. In this chapter, we discuss serum uric acid as a biomarker in diabetes, its complications, and its relationship with HbA1c and eGFR.

Keywords

Serum uric acid · Diabetes · Biomarkers · Diabetic complications · eGFR · HbA1c · Diabetes risk · Diabetic retinopathy · Diabetic nephropathy · Prediabetes

Abbreviations

BCAA	Branched-chain amino acids
BMI	Body mass index
CRP	C-reactive protein
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase-1
HDL-C	High-density lipoprotein cholesterol
IL-6	Interleukin-6
LDL-C	Low-density lipoprotein cholesterol
NO	Nitric oxide
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SUA	Serum uric acid
TNFα	Tumor necrosis factor-α

Introduction

Biomarkers are surrogate indicators of disease risk, presence of the disease, and its progression. Even as proteins are extensively studied as biomarkers in chronic diseases, metabolites are gaining importance. Metabolites are end products of cellular metabolism. These are small molecules and are relatively easier to measure in biological samples than proteins. The human metabolome database contains

~115,000 entries (Wishart et al. 2018). The perturbation in metabolism starts early in many chronic diseases. Identifying appropriate metabolic markers will help in detecting the disease in a very early stage. Metabolic diseases such as diabetes have a very long period of metabolic abnormalities before the onset of the disease. Therefore, metabolic markers can be reliably applied for predicting the future risk of diabetes and its current status, response to treatments, and prognosis.

The traditional risk factors such as age, gender, BMI, fasting glucose levels, and insulin levels provide a very good risk estimate for predicting diabetes mellitus. However, these markers are useful only in the presence of overt metabolic changes, and they fail to detect the metabolic abnormalities that usually begin several years before the onset of diabetes. Changes in glycemia and insulin levels occur long after the initial metabolic abnormalities began. Therefore, blood glucose levels and insulin levels are not good markers for the early detection of the disease. Diabetes is usually preceded by prediabetes for years. Metabolic alterations that pave the way for prediabetes occur many years before prediabetes. Lifestyle changes and specific prevention programs are reported to prevent or delay diabetes and reverse the prediabetic condition in many patients. Thus, to apply preventive strategies, there is a need to identify biomarkers other than blood glucose and insulin that can reliably predict the disease risk at a very early stage.

Diabetes is a progressive disease, and it becomes difficult to control the disease over time. Sustained hyperglycemia damages the tissues and causes complications in multiple organs. Microvascular (retinopathy and nephropathy) and macrovascular (cardiovascular and cerebrovascular diseases) are some of the most common complications of diabetes. Since the tissue damages cannot be reversed, early disease control is essential to prevent diabetic complications. Even though glycemia is a significant risk factor for diabetic complications, it can change quickly and is unreliable for predicting the future risk of developing complications. Further, it cannot indicate the extent of metabolic changes and the damage to tissues. Therefore, identifying molecules involved in the abnormal cellular metabolic alterations will be the key to knowing about the disease progression and the extent of damage happening. Metabolic markers that are highly sensitive and specific are needed to detect the early risk of diabetes and its complications.

Metabolic Markers in Diabetes

Associations between metabolic pathways and the disease have been explored to find suitable metabolic markers. The metabolomic approach provides an excellent opportunity to explore all the associated metabolites with the disease condition. It simultaneously measures many metabolites in the given biological sample. Mass spectrometry and nuclear magnetic resonance spectroscopy are the two commonly used technologies for metabolomic studies. These techniques are often coupled with a separation technique to yield a reliable and accurate measure of different metabolites. In recent years, the application of metabolomics to type 2 diabetes has provided a better understanding of different pathophysiological pathways and the

identification of several novel biomarkers. Amino acids are the most common metabolites found associated with diabetes. Protein and carbohydrate metabolism are interlinked at the molecular level. The metabolites of amino acids form the fuel for gluconeogenesis. The precursors of gluconeogenesis, such as oxaloacetate and pyruvate, are produced from the deamination of amino acids (Bender 2012). Free amino acids produced from the de novo biosynthesis using the Krebs cycle intermediates modulate the glucagon and insulin secretion. Overactive glucagon-induced gluconeogenesis has been found in diabetic conditions (Girard 2017). Therefore, the excess and free amino acids can be utilized as good metabolite markers.

Serum amino acid levels were linked to the incidence of type 2 diabetes. Several longitudinal cohort studies have linked altered baseline amino acid levels to the increased incidence of diabetes over time (Wang et al. 2011; Stancáková et al. 2012; Bender 2012; Palmer et al. 2015; Tillin et al. 2015; Yamakado et al. 2015; Chen et al. 2016, 2019). Branched-chain amino acids (BCAA) are directly implicated in diabetes risk (Nawaz and Siddiqui 2020). Increased levels of BCAAs were reported to increase insulin resistance in several studies. BCAAs are believed to cause insulin resistance by increasing the phosphorylation of insulin receptors through the activation of mammalian targets of rapamycin complex 1 (Yoon 2016). Higher levels of BCAAs are associated with older age, male sex, and metabolic syndrome, as well as with obesity, cardiovascular risk, dyslipidemia, hypertension, and uric acid (Hu et al. 2016). More than 50 amino acids and their metabolites are reported to be associated with increased type 2 diabetes (Hameed et al. 2020).

Central obesity is associated with insulin resistance, metabolic abnormalities, and type 2 diabetes. Abdominal fat deposition seen in central obesity causes lipid metabolism alterations and leads to dyslipidemia (Papaetis et al. 2015). The alteration in lipid metabolism occurs several years before the onset of dysglycemia. Therefore, the classical biomarkers of dyslipidemia such as hypertriglyceridemia, increased low-density lipoprotein cholesterol (LDL-C), and reduced high-density lipoprotein cholesterol (HDL-C) are also able to predict the risk of diabetes (Habiba et al. 2016). The lipidomic biomarkers of diabetes include glycerolipids, phospholipids, acylcarnitines, and free fatty acids. Several prospective and cross-sectional studies have found lipid metabolites such as triacylglycerols, cholesterol esters, glycerophospholipids, diacyl phosphatidylcholines, alkyl acyl phosphatidylcholines, lysophosphatidylcholines, phosphatidylethanolamines, alkyl acyl phosphatidylethanolamines, lysophosphatidylethanolamines, sphingomyelins, and ceramides as biomarkers predicting the risk of diabetes and prediabetes in comparison to healthy individuals (Hameed et al. 2020).

Uric Acid

Uric acid, an organic compound, is the end product of purine metabolism in the liver. The source of endogenous purines is nucleic acids adenine and guanine. Enzymatic degradation of these nucleic acids results in the production of uric acid. Various animal proteins from the diet constitute the source of exogenous purines. Fructose

from fruits and added sugar also contributes to the production of uric acid. However, the impact of dietary purines on serum uric acid (SUA) levels is significantly less. A purine-rich diet will cause an increase in the SUA levels by 1–2 mg/dl only (Burini 2012). It is excreted mainly via the kidneys and one-third through the intestine. It is a weak acid and circulates as monosodium salt. The SUA levels increase with age and are higher in men. Normal SUA levels are 2.5–7.0 mg/dl in men and 1.5–6.0 mg/dl in women. The levels increase in postmenopausal women to reach the range seen in men.

Uric acid has low solubility in water and plasma (6.8 mg/dl). Therefore, when the blood uric acid levels approach the upper limit of the normal range, it will form insoluble urate crystals. However, plasma protein binding increases the solubility of urate in the blood. Classically, the hyperuricemic condition is implicated in the development of gout and renal stones. Recent studies have indicated that uric acid is associated with several characteristics of cardiometabolic diseases. It is thought to be actively involved in the pathological processes of insulin resistance, hypertension, cardiovascular disease, and chronic kidney disease. Altered SUA levels may indicate changes in the metabolic state. Therefore, SUA level can be a potential biomarker of cardiometabolic diseases. We will discuss the role of uric acid as a biomarker in diabetes and associated conditions in this chapter.

Uric Acid in Diabetes Pathophysiology

SUA levels in healthy adults remain relatively constant. However, changes in its production and excretion may alter the levels and lead to increased or decreased uric acid levels in the body fluids. Hyperuricemic conditions are implicated in the pathogenesis of diabetes. Increased uric acid level causes oxidative stress, leads to the activation of inflammatory processes, inhibits insulin pathway, and increases hepatic glucose production and dysfunction and death of pancreatic beta cells (Fig. 1). Higher uric acid levels are associated with the upregulation of inflammatory cytokines. SUA is positively correlated with acute-phase proteins such as C-reactive protein (CRP), fibrinogen, ferritin, component C3, and erythrocyte sedimentation rate in a non-diabetic cohort. It was further confirmed that uric acid stimulated the expression of CRP, fibrinogen, ferritin, and component C3 in a dose-dependent manner (Spiga et al. 2017). In a large population-based cross-sectional study, SUA levels were found to be associated with IL-6, TNF-α, and CRP. These inflammatory cytokines play a central role in the pathophysiology of type 2 diabetes (Tsalamandris et al. 2019).

The production of uric acid generates reactive oxygen species (ROS) during the process. Uric acid is a potent antioxidant in the plasma. However, it increases the ROS in vascular tissue and acts as a prooxidant. Uric acid is shown to contribute to oxidative stress. It increased the reactive oxygen species production under physiological conditions independent of xanthine oxidase, an oxidative enzyme that catalyzes the production of uric acid (Kurajoh et al. 2021). Under hyperuric conditions, the oxidative stress and inflammatory response are related to the uric acid-induced

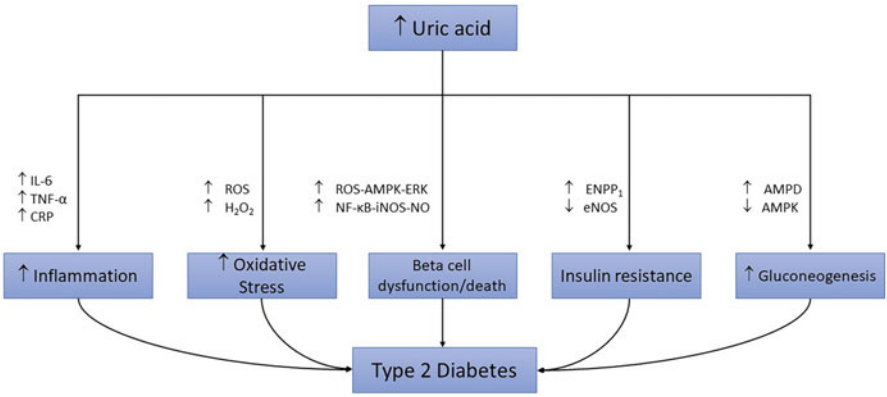

Fig. 1 Role of uric acid in the pathophysiology of type 2 diabetes mellitus. NF-κB-iNOS-NO, nuclear factor kappa B (NF-κB)-iNOS-NO signaling pathway; ROS-AMPK-ERK, reactive oxygen species (ROS)-AMP-activated protein kinase (AMPK)-extracellular signal-regulated kinase (ERK) signaling pathway; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; AMPD, adenosine monophosphate dehydrogenase; AMPK, adenosine monophosphate protein kinase

oxidative stress (Zhou et al. 2018). The uric acid-mediated oxidative stress has been implicated in activating inflammatory factors, lipid peroxidation, DNA damage, and cell death (Yu et al. 2010). Uric acid is also reported to cause endothelial dysfunction through oxidative stress. It is thought to be mediated through the activation of the renin-angiotensin system (Yu et al. 2010).

Pathophysiology of type 2 diabetes mainly revolves around the development of insulin resistance and beta-cell dysfunction. SUA concentrations have been shown to be associated with insulin resistance, impaired insulin secretion, and beta-cell death (Ghasemi 2021). Initially, it was thought high uric acid levels did not affect beta-cell function. But, a recent animal study has shown that hyperuricemia promotes pancreatic beta-cell death (Lu et al. 2020). In a cross-sectional study, SUA levels were associated with insulin resistance and impaired insulin secretion. A SUA level of 5.5 mg/dl identified the people with insulin resistance (Martínez-Sánchez et al. 2021). In another cross-sectional study, similar results were obtained. SUA levels were independently associated with impaired fasting glucose and insulin resistance (Yu et al. 2021). High uric acid concentrations decrease the insulin-induced nitric oxide (NO) synthesis in the endothelium by reducing eNOS expression and direct inactivation of NO (Bahadoran et al. 2021). The uric acid-induced endothelial insulin resistance eventually leads to the development of systemic insulin resistance. Further, uric acid is reported to interfere with insulin signaling at the receptor level directly. Uric acid activates ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), a transmembrane glycoprotein that deactivates insulin receptors. In vitro studies have shown that uric acid induced the binding of ENPP1 to the insulin receptor (Tassone et al. 2018).

The effect of uric acid on basal insulin secretion and glucose-induced secretion is not consistent. It inhibited glucose-induced insulin secretion in the in vitro studies

involving rat and mouse pancreatic islets and pancreatic beta-cell lines. However, uric acid increased the glucose-induced insulin secretion in the perfused rat pancreas and in type 2 diabetes patients under hyperglycemic clamp conditions (Ghasemi 2021). These studies reflect the acute effect of uric acid on insulin secretion by beta cells. But continued high SUA levels cause impaired insulin signaling and beta-cell dysfunction. Therefore, prolonged exposure to high uric acid levels will ultimately lead to impaired glucose metabolism and increased blood glucose levels.

Interestingly, the association between SUA levels and blood glucose has not been consistent among people with diabetes. The relationship between glycemia and uric acid levels varies according to the other confounding factors. Hyperglycemia causes hyperfiltration in the kidneys and can result in the increased excretion of uric acid (Wei et al. 2016). High urinary excretion of glucose increases the urinary excretion of uric acid and leads to lower SUA levels (Qin et al. 2021). On the other hand, low eGFR levels or decreased renal function, a condition often found in patients with diabetes, will affect the clearance of uric acid. Our recent cross-sectional study on patients with type 2 diabetes confirmed the influence of eGFR on the relationship between SUA levels and glycemia (Rafiullah et al. 2020).

Uric Acid as a Biomarker in Diabetes

The relationship between SUA levels and diabetes depends on the period of assessment. Several studies have evaluated the use of SUA levels as a predictor of diabetes risk in healthy individuals, a predictor of diabetes prognosis in patients with diabetes, and a predictor of diabetic complications in patients with diabetes (Table 1). SUA concentrations vary throughout the time according to the current clinical condition and different comorbidities. SUA levels are high in the early stages of impaired glucose metabolism but come down once the blood glucose levels reach the diabetic range. Age, gender differences, and renal function influence the relationship between SUA with diabetes.

Serum Uric Acid as a Predictor of Diabetes Risk

The role of uric acid in the development of abnormal glucose metabolism by causing insulin resistance, impaired insulin secretion, and beta-cell dysfunction suggests it could be an early indicator of diabetes risk. The SUA levels are often found elevated during the early phase of impaired glucose metabolism. A meta-analysis of prospective cohort studies found strong evidence for SUA levels as a predictor of diabetes risk (Lv et al. 2013). The analysis included ~32,000 participants from 8 prospective cohorts. The adjusted relative risk of developing type 2 diabetes for the highest uric acid level group was 1.56% (95% CI, 1.39–1.76).

Further, it was found that the risk of type 2 diabetes increased by 6% for each 1 mg/dl increase in the SUA level. The adjustment included all the components of metabolic syndrome. Another meta-analysis found a non-linear relationship between the SUA

Table 1 Association of serum uric acid level with diabetes and its complications

Study	Study type	Population	Variables controlled	Results
(Lv et al. 2013)	Meta-analysis of prospective studies	Mixed	Age, sex, BMI, blood pressure, HDL, TG, plasma glucose, smoking, alcohol consumption, and physical activity	RR for DM in highest vs lowest quartile, 1.56 (95% CI, 1.39–1.76)
(Jia et al. 2013)	Meta-analysis of cohort studies	Mixed	Hypertension, alcohol use, BMI, TG, and TC	5.5 mg/dl of SUA: RR for DM 1.25 (95% CI, 1.16–1.35) 6.5 mg/dl of SUA: RR for DM 1.43 (95% CI, 1.31–1.55) 7.5 mg/dl of SUA: RR for DM 1.50 (95% CI, 1.38–1.63) 8.5 mg/dl of SUA: RR for DM 1.49 (95% CI, 1.34–1.67)
(Bombelli et al. 2018)	Retrospective cohort	Italian	Age, sex, baseline blood glucose level, BMI, diuretic use, smoking habits, and alcohol consumption	RR for IFG, 1.26 (95% CI,1.06–1.5)
(Wu et al. 2020)	Population-based prospective cohort	Taiwanese	Age, sex, cigarette smoking, alcohol drinking, regular exercise level, body mass index, waist circumference, systolic blood pressure, and levels of glucose and low-density lipoprotein cholesterol	Highest vs lowest quartile HR for DM 1.94 (1.05–4.05)
(Zhang et al. 2016)	Prospective cohort	Chinese	Age, sex, BMI, smoking status, drinking status, sBP ⩾140 mmHg, or dBP ⩾90 mmHg, or history of hypertension, TC ⩾5.17 mmol/L, or TG ⩾1.7 mmol/L, or LDL ⩾3.37 mmol/L, or history of hyperlipidemia), and family history of CVD, hypertension, hyperlipidemia, and diabetes	HR for IFG: Quintiles 2 vs 1, 1.14 (95% CI, 1.03, 1.25) Quintiles 3 vs 1, 1.12 (95% CI, 1.02, 1.23) Quintiles 4 vs 1, 1.20 (95% CI, 1.08, 1.32) Quintiles 5 vs 1, 1.22 (95% CI, 1.10, 1.36)

(continued)

Table 1 (continued)

Study	Study type	Population	Variables controlled	Results
(Lehto et al. 1998)	Population-based prospective cohort		Age, gender, smoking, total cholesterol, hypertension, BMI, serum total triglycerides, HDL, plasma glucose, previous history of stroke, use of diuretics, and known duration of diabetes	Hyperuricemic vs normouricemic HR for stroke 1.91 (95 CI%, 1.24 to 2.94)
(Seghieri et al. 2002)	Cross-sectional	Finnish	Sex, age, blood pressure, serum creatinine, plasma glucose, lipids, presence of coronary heart disease, and atrial fibrillation	Highest vs lowest quartile OR for stroke 1.32 (95% CI 1.07–1.41)
(Du et al. 2017)	Meta-analysis	Mixed	Not available	Ratio of means for risk of stroke 1.29 (95% CI, 1.26–1.31)
(Kuwata et al. 2017)	Prospective cohort	Japanese	Age, BMI, alcohol drinking, sBP, dBP, HDL, triglycerides, eGFR, HbA1c, duration of diabetes, anti-hyperuricemia drug use, ACEI use, ARB use, diabetes therapy, smoking, and history of cardiovascular disease	HR for DR in males: Q2 vs 1, 1.97 (95% CI, 1.14–3.41) Q3 vs 1, 1.92 (95% CI, 1.18–3.13) Q4 vs 1, 2.17 (95% CI, 1.40–3.37)
(Lee et al. 2014)	Prospective cohort	Taiwanese	Age, sex sBP, dBP, eGFR, HDL, TG, BMI, and waist circumference	HR for worsening of DR: Q3, 2.57 (95% CI, 1.30–5.08) Q4, 3.66 (96% CI, 1.92–7.00)
(Hu et al. 2021a)	Cross-sectional	Chinese	Age, sex, BMI, hypertension, SBP, diabetes duration, HbA1c, chronic kidney disease, total cholesterol, HDL, LDL, and urinary uric acid excretion	OR for vision-threatening DR in Q3, 1.60 (95% CI, 1.07–2.39) Q4, 2.05 (95% CI, 1.37–3.08)
(Bartáková et al. 2016)	Prospective cohort	Caucasian	Not available	HR for DKD 2.14 (96%CI, 1.40–3.26) HR for DKD progression 1.54 (1.07–2.22)

(continued)

Table 1 (continued)

Study	Study type	Population	Variables controlled	Results
(Hayashino et al. 2016)	Prospective cohort	Japanese	Age, gender, BMI, smoking, sBP, dBP, hs-CRP, HDL, LDL, TG, serum creatinine, eGFR, ACEI use, anti-hyperuricemic drug use, ARB use, HbA1c level, past history of cardiovascular disease, and diabetic retinopathy	HR for the progression from microalbuminuria to macroalbuminuria, when compared to Q2: Q1, 2.17 [95% CI, 1.15–4.08) Q3, 3.04 (95% CI, 1.67–5.53) Q4, 3.56 (95% CI, 1.83–6.93)
(Gu et al. 2017)	Retrospective longitudinal study	Chinese	Age, gender, BMI, sBP, HbA1c, LDL, eGFR, use of statins, ACEI, or ARB	HR for CKD in Q4, 2.615 (95% CI, 1.040–6.574)

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CKD, chronic kidney disease; CRP, C-reactive protein; dBP, diastolic blood pressure; DKD, diabetic kidney disease; DM, diabetes mellitus; DR, diabetic retinopathy; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; HR, hazard ratio; IFG, impaired fasting glucose; LDL, low-density lipoprotein cholesterol; OR, odds ratio; Q1, Q2, Q3, and Q4, quartiles 1, 2, 3, and 4, respectively; RoM, ratio of means; RR, relative risk; sBP, systolic blood pressure; SD, standard deviation; SUA, serum uric acid; TC, total cholesterol; TG, triglycerides

levels and the incidence of type 2 diabetes. It included data from 12 studies involving ~62,000 participants. The relative risk of impaired fasting glucose and type 2 diabetes in the highest SUA level group was found to be 1.54 (95% CI, 1.41–1. 68) (Jia et al. 2013).

There are many large prospective studies that have been published after the meta-analysis in support of the use of uric acid as a predictor of diabetes risk. In a prospective cohort study of 3200 Italian individuals, baseline SUA level was associated with new onset of diabetes. There was a 29% increase in the risk of impaired fasting glucose (Bombelli et al. 2018). In a population-based prospective cohort study in Taiwan, the baseline SUA concentration was associated with diabetes risk independent of other known risk factors. The association was found across different age groups and genders (Wu et al. 2020). But conflicting results have been reported in the gender differences of the association of SUA with the incidences of diabetes (Chen et al. 2018; Lou et al. 2020). A study investigating the gender differences in the association of uric acid with diabetes risk found that older women, especially those aged >48 years, have a higher risk of diabetes. It appears to be a confounding effect of estrogen with SUA levels in the younger women, as estrogen increases the excretion of uric acid. It also has been demonstrated that postmenopausal women have increased SUA levels (Liu et al. 2018). Therefore, the menstrual state of women needs to be taken into account while assessing the association of SUA level with metabolic parameters. SUA levels could predict the

future incidence of prediabetes as well. After 3 years of follow-up, mean SUA levels were predictive of prediabetes in a cohort of ~39,000 normoglycemic individuals (Zhang et al. 2016).

Serum Uric Acid, Blood Glucose Level, and HbA1c

SUA levels showed an inconsistent relationship with glycemic parameters. Under hyperglycemic conditions, the increased urinary glucose excretion increases uric acid's renal clearance and leads to lower SUA concentrations. It results in an inverse relationship between blood glucose and uric acid levels (Qin et al. 2021). Impaired renal function nullifies this effect and reverses the relationship between blood glucose and uric acid levels (Rafiullah et al. 2020). Thus, the association of SUA concentration with blood glucose level is dependent on the glycosuria and extent of renal function. Many studies have not considered these factors into account and, thus, reported conflicting outcomes. Evidence shows that SUA levels are elevated during prediabetes and fall once the glucose levels reach the diabetic range. SUA concentration negatively correlated with fasting plasma glucose and HbA1c in people with diabetes and positively correlated with fasting plasma glucose and HbA1c in normal individuals (Wei et al. 2016). High SUA levels were inversely associated with diabetes mellitus in the Third National Health and Nutrition Examination Survey cohort (Bandaru and Shankar 2011). People with a longer duration of diabetes tend to have lower SUA levels and an inverse association with fasting blood sugar and HbA1c. However, in newly diagnosed type 2 diabetic patients, the inverse association was seen only in patients who had high insulin levels (Cui et al. 2016).

Uric Acid in Diabetic Complications

Oxidative stress and endothelial dysfunction play an important role in the development of different diabetic complications. Uric acid induces oxidative stress and endothelial dysfunction and worsens insulin resistance by interfering with insulin-stimulated glucose uptake in the skeletal muscles (Tassone et al. 2011). Elevated uric acid levels cause increased oxidative stress, inflammation, endothelial dysfunction, activation of the renin-angiotensin-aldosterone system (RAAS), and dyslipidemia (Fig. 2). These factors individually or collectively lead to different complications of diabetes (Kanbay et al. 2013). A genetic Mendelian randomization study demonstrated the causal relationship between uric acid and diabetic macrovascular complications (Yan et al. 2016). Therefore, a higher level of SUA is likely to accelerate the development of diabetic complications and be associated with the risk of developing diabetic complications. Several studies have established the use of SUA levels to predict the future risk of diabetic complications (Table 1). A meta-analysis showed elevated SUA levels as an independent predictor of vascular complications and mortality in patients with type 2 diabetes (Xu et al. 2013).

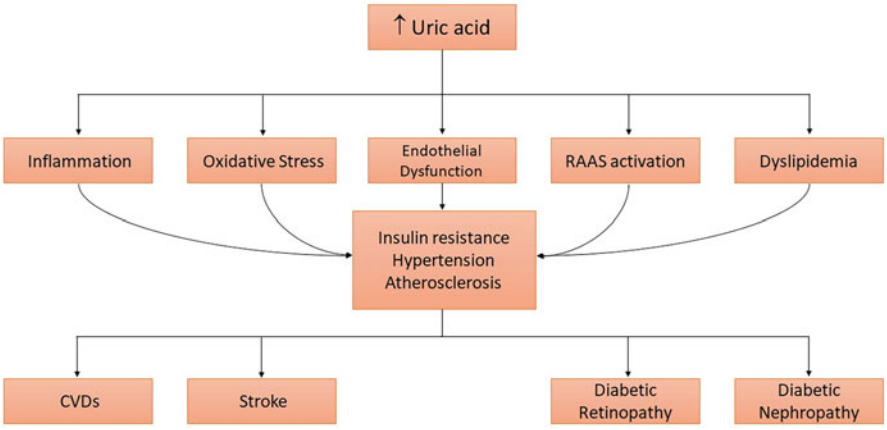

Fig. 2 Role of uric acid in the development of diabetic complications. CVD, cardiovascular disease; RAAS, renin-angiotensin-aldosterone system

Uric Acid in Macrovascular Complications

The association of SUA levels with cardiovascular risk factors is well known. People with hyperuricemia and gout are shown to be associated with subclinical atherosclerosis and an increased risk of cardiovascular events (Gagliardi et al. 2009). Elevated SUA concentrations have been linked to a higher risk of cardiovascular events and mortality in the general population. Large meta-analyses have consistently demonstrated the association of SUA level and cardiovascular diseases risk (Braga et al. 2016; Wang et al. 2016). However, in people with diabetes, the association between SUA and the risk of cardiovascular disease has not been consistent. In the Verona Diabetes Study cohort, SUA levels are independently associated with increased risk of cardiovascular mortality in type 2 diabetes (Zoppini et al. 2009). But the Framingham Heart Study found SUA levels were not associated with coronary heart disease after adjusting other cardiovascular risk factors (Culleton et al. 1999). The Fremantle Diabetes Study produced similar results. It showed serum uric level was not an independent predictor of cardiovascular disease or all-cause mortality in type 2 diabetes patients (Ong et al. 2010). Population-based Casale Monferrato Study also concluded that SUA level is not an independent risk factor for cardiovascular mortality (Panero et al. 2012). Therefore, uric acid is not a good predictor of cardiovascular diseases in people with diabetes.

SUA can be used as a biomarker to identify patients with high ischemic stroke risk. It is known to be a predictor of stroke in patients with type 2 diabetes for a long time (Lehto et al. 1998). Increase in SUA concentrations is selectively associated with ischemic stroke in patients with type 2 diabetes compared to non-diabetic individuals. The association remained significant even after adjusting gender, age, blood pressure, plasma glucose, serum creatinine, lipid profile, and presence of

cardiovascular disease (Seghieri et al. 2002). In a meta-analysis of observational studies, type 2 diabetes patients with cerebral infarction had 26% higher SUA levels than those without cerebral infarction (Du et al. 2017). Interestingly, a large cross-sectional study on type 2 diabetes patients in China found that the association between SUA levels and incidence of ischemic stroke depends on the patients' age. The SUA levels were independently and positively associated with ischemic stroke in patients aged <60 years. But the association reversed in patients aged ≥60 years (Wang et al. 2017).

Uric Acid in Microvascular Complications

Diabetic microvascular complications like diabetic retinopathy and diabetic nephropathy are associated with elevated SUA levels. Studies in animal models have found that hyperuricemia promotes the development of diabetic retinopathy by increasing the inflammation of the retina and by increasing the Notch signaling pathway (Zhu et al. 2018). Several observational and prospective clinical studies have established the relationship between elevated SUA levels and the risk of diabetic retinopathy (Table 1). A cross-sectional study of ~18,000 Chinese individuals found an independent association of SUA level with diabetic retinopathy (Cui et al. 2017). In a prospective cohort of Japanese individuals (diabetes distress and care registry at Tenri, DDCRT), newly developed diabetic retinopathy was associated with higher quartiles of SUA levels in men after 2 years of follow-up. The association was not found in women (Kuwata et al. 2017). Gender difference in uric acid metabolism is one of the major confounding factors in these studies. The relationship of SUA with diabetes and its complications in women depends upon the menopausal state.

The presence of renal impairment appears to influence the association between SUA and diabetic retinopathy. In a large cross-sectional study of ~3,000 patients, SUA was associated with diabetic nephropathy and not with diabetic retinopathy (Xia et al. 2020). Similar results were obtained in another cross-sectional study (Hu et al. 2021b). In addition to its association with incidence, SUA levels also predicted the worsening of diabetic retinopathy. In a 3-year prospective study, SUA concentration was associated with worsening of non-proliferative diabetic retinopathy. Patients in the third and fourth quartiles of SUA concentration had significant hazard ratios for worsening of diabetic retinopathy when compared with patients in the first quartile of SUA levels (Lee et al. 2014). A similar finding was observed in a cross-sectional study as well. High SUA levels were associated with more serious diabetic retinopathy (Chen et al. 2020). In another large cross-sectional study, higher SUA levels were associated with an increased risk of vision-threatening diabetic retinopathy (Hu et al. 2021a).

The correlation between diabetic nephropathy and SUA is a well-known association. As SUA is known to activate the renin-angiotensin system, it may be possible to be involved directly in the pathogenesis of diabetic nephropathy. It is even suggested to mediate diabetic nephropathy (Jalal et al. 2011). Hyperuricemia is

independently associated with increased risk of incident chronic kidney disease in patients with type 2 diabetes (Zoppini et al. 2012). A longitudinal study of type 2 diabetes patients from the Italian Association of Clinical Diabetologists Network database found that mild hyperuricemia was associated strongly with chronic kidney disease. However, SUA is associated with albuminuria only when the eGFR <60 ml/min/1.73 m^2 (De Cosmo et al. 2015). SUA level within the high normal range is independently associated with diabetic kidney disease in a cross-sectional analysis of ~3200 Chinese individuals. SUA levels showed a positive correlation with albuminuria and creatinine levels, whereas a negative relationship with eGFR (Yan et al. 2015). In patients with diabetes and preserved kidney function at baseline, high normal SUA levels predicted the progression to stage 3 of the chronic kidney disease (Kim et al. 2014). Baseline hyperuricemia predicted the progression of diabetic kidney disease in a prospective study that followed the patients for 15 years. In the same study, the optimal cut-off value of SUA concentration in patients with type 2 was found to be 6.3 mg/dl in men and 5.2 mg/dl in women (Bartáková et al. 2016).

A recent study found no significant difference between the SUA levels and the risk of progression of diabetic nephropathy to end-stage renal disease. The study included patients with biopsy-confirmed diabetic nephropathy at baseline and followed them for 3 years (Zou et al. 2021). Another study in a Japanese cohort found that the SUA levels showed a U-shaped risk for progression from microalbuminuria to macroalbuminuria. Patients in the low and high quartiles of SUA concentration showed a significantly increased risk of the progression of diabetic nephropathy (Hayashino et al. 2016). Gu et al. demonstrated that SUA-to-creatinine ratio could be a superior predictor of chronic kidney disease among patients with type 2 diabetes (Gu et al. 2017). Since renal function influences the SUA levels, the renal function-normalized value of SUA concentration showed a better prediction.

eGFR and Serum Uric Acid Levels

Renal impairment affects uric acid excretion and can thus increase the serum concentration of the uric acid (Wang et al. 2021). Consequently, the eGFR levels show a negative relationship with SUA concentrations. Patients with high SUA had a rapid decline in the eGFR over 5 years. But the association was only marginally significant after adjustment for covariates (Le et al. 2021). SUA levels >6.2 mg/dl and > 6.5 mg/dl identified early nephropathy and decline in eGFR, respectively (Yan et al. 2015). A 5-year follow-up cohort study found that the SUA and eGFR decline were associated in men but not in women (Wang et al. 2018). In a 4-year follow-up cohort of ~14,000 patients with type 2 diabetes, a decline in eGFR was associated with high SUA concentrations (De Cosmo et al. 2015). A meta-analysis assessing the association between SUA and eGFR decline among patients with type 2 diabetes in prospective cohort studies demonstrated that a 1.68 mg/dl increase in SUA level is associated with a 33% increased risk of eGFR decline (Wang et al. 2018). The association between SUA and diabetic kidney disease depended on the eGFR levels. SUA is associated with albuminuria only in when the eGFR <60 ml/min/1.73 m^2

(De Cosmo et al. 2015). In our recent work, we showed that the association of SUA with different variables depended on the eGFR status of patients (Rafiullah et al. 2020). Hyperglycemic conditions also influence the SUA levels by interfering with the urinary excretion of uric acid. But changes in eGFR directly affect the excretion of uric acid and hence change the relationship of the SUA concentration with other patient variables. Therefore, the association of SUA concentration with diabetes-related variables is influenced by the eGFR status of the patients.

Applications to Prognosis and Other Diseases or Conditions

Several large epidemiological studies have demonstrated that SUA levels can predict the diabetes risk in people with prediabetes or normoglycemia. SUA levels have been shown to be elevated constantly during the early phase of impaired glucose metabolism. However, the association of SUA and blood glucose level after the development of diabetes becomes unreliable. Many studies have shown an inverse association between SUA and glycemic parameters. As a result, the SUA levels will not be helpful to assess the diabetes prognosis. Instead, the association of SUA concentration with different diabetic complications can be used to predict and evaluate the prognosis of the complications. Elevated SUA level independently predicted vascular complications and mortality in people with diabetes. It can predict the incidence of cardiovascular diseases, ischemic stroke, and diabetic retinopathy and nephropathy. Increased SUA concentrations can be an indication of progression of diabetic nephropathy.

Applications to Prognosis

In this chapter, the role of SUA concentration as a biomarker to predict the risk of diabetes and its complications has been reviewed. SUA levels demonstrated a significant correlation with the risk of developing type 2 diabetes (Lv et al. 2013; Jia et al. 2013). Evidence shows that every 1 mg/dl increase results in a 6% increased risk for type 2 diabetes. The association of SUA is independent of other known risk factors for diabetes. In addition to the risk of diabetes, SUA levels are also useful in predicting the risk of diabetic complications (Xu et al. 2013). Hyperuricemia independently predicted the risk of chronic kidney disease in people with diabetes (Cui et al. 2017). Baseline uric acid levels predicted the progression of diabetic kidney disease. The optimum cut-off value for SUA was found to be 6.3 mg/dl in men and 5.2 mg/dl in women (Bartáková et al. 2016).

Applications to Other Diseases or Conditions

SUA is involved in the pathophysiology of metabolic abnormalities that lead to the onset of type 2 diabetes. Even though this is not the case with type 1 diabetes, uric

acid might play a role in some complications. Uric acid contributes to the development of hypertension and chronic kidney disease independent of the diabetes status of the people. Therefore, SUA concentration may be used to predict diabetic nephropathy in patients with type 1 diabetes. Baseline elevated uric acid levels predicted subsequent incidence of macroalbuminuria (Hovind et al. 2009). A 4.11 m./min decrease in eGFR was observed for every 1 mg/dl increase in SUA concentration in individuals with type 1 diabetes (Pizarro et al. 2018). For every 1 mg/dl increase in SUA concentration level at baseline, there was an 80% increase in micro- or macroalbuminuria at 6 years (Jalal et al. 2010).

Mini-Dictionary of Terms

- **Prediabetes**. A condition that precedes diabetes, characterized by impaired fasting glucose (fasting plasma glucose levels 100 mg/dl to 125 mg/dl) and/or an oral glucose tolerance test 2 h blood glucose levels 140–199 mg/dl or an HbA1c level 5.7–6.4%.
- **Hyperuricemia**. SUA levels $>$ 7.0 mg/dl in men and $>$ 6.0 mg/dl in women.
- **Xanthine oxidase**. An oxidative enzyme that catalyzes the production of uric acid.
- **ENPP1**. Ectonucleotide pyrophosphatase/phosphodiesterase-1 is a transmembrane glycoprotein that deactivates insulin receptor.
- **Renal hyperfiltration**. A condition associated with early stages of kidney disease, characterized by increased glomerular filtration rates above the normal values.

Key Facts of Serum Uric Acid and Metabolic Markers in Diabetes: eGFR, HbA1c, and Beyond

Key Facts of Uric Acid and Diabetes

- Uric acid is involved in the pathophysiology of type 2 diabetes.
- Elevated SUA levels are found in people with prediabetes.
- SUA levels predict the risk of diabetes in healthy individuals.
- SUA and glycemic parameters in diabetic individuals are negatively associated.
- Measuring SUA will not be helpful to predict the prognosis of diabetes.

Key Facts of Uric Acid and Diabetic Complications

- Uric acid is involved in the development of diabetic complications.
- SUA level is not a good predictor of cardiovascular diseases in people with diabetes.

- SUA can be used as a biomarker to identify patients with high ischemic stroke risk.
- SUA levels are associated with worsening of diabetic retinopathy.
- High normal SUA levels predicted the progression of diabetic nephropathy.
- SUA levels are associated positively with albuminuria and negatively with eGFR.

Summary Points

- SUA concentrations have been shown to be associated with insulin resistance, impaired insulin secretion, and beta-cell dysfunction.
- SUA levels are found to be high early stages of impaired glucose metabolism but come down once the blood glucose levels reach the diabetic range.
- Age, gender differences, and renal function influence the relationship between SUA with diabetes.
- Elevated SUA level is an early indicator of diabetes risk.
- SUA concentration negatively correlated with fasting plasma glucose and HbA1c in people with diabetes.
- SUA level can predict the future risk of diabetic complications.
- SUA can be used as a biomarker to identify patients with high ischemic stroke risk. It is associated with worsening diabetic retinopathy.
- High normal SUA levels predicted the progression of diabetic nephropathy.
- SUA levels are associated positively with albuminuria and negatively with eGFR.

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Urinary Interleukins and Kidney Damage in Diabetes

11

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Contents

Introduction 263
Inflammation and Diabetic Kidney Disease 264
Urinary Interleukins 267
Interleukin 1 267
Interleukin 6 268
Interleukin 8 269
Interleukin 10 270
Interleukin 18 270
Potential Applications to Prognosis, Other Diseases, or Conditions 271
Mini-dictionary of Terms 272
Key Facts of Diabetes, Kidney Damage, and Urinary Interleukins 272
Summary Points 273
References 273

Abstract

Diabetic kidney disease (DKD) is a progressive microvascular complication of diabetes mellitus. In the natural history of DKD, insistent microalbuminuria progresses to overt proteinuria, a gradual decline in the glomerular filtration rate, and, eventually, renal failure. Currently, urinary albumin excretion (UAE) is widely used to indicate early phases of DKD, although it is limited by the fact that structural damage might precede albumin excretion. This observation points

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to the need to study new urinary biomarkers that are more sensitive and specific than the UAE, which may allow the early detection of DKD. In addition, an increasing number of clinical and experimental studies have pointed out that inflammation contributes to the development and progression of DKD and that urinary excretion of inflammatory markers, including the interleukins, may be useful as biomarkers in assessing renal damage. Therefore, this chapter revised the general aspects of inflammation-related processes involved, specifically the role of interleukins in the pathophysiology of DKD. Furthermore, this chapter describes how urinary interleukin concentrations, especially IL-1, IL-6, IL-8, IL-10, and IL-18, may be related to early progressive renal function decline in diabetes.

Keywords

Diabetes · Kidney damage · Inflammation · Interleukins · Urine

Abbreviations

AER	Albumin excretion rate
AGEs	Advanced glycation end products
AKI	Acute kidney injury
DAMPs	Damage-associated molecular patterns
DKD	Diabetic kidney disease
DM	Diabetes mellitus
ECM	Extracellular matrix
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL-1	Interleukin 1
IL-1R	Receptor of IL-1
IL-1Ra	IL-1 receptor antagonist
JAK/STAT	Janus kinase-signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase
mIL-6R	Membrane IL-6 receptor
MMPs	Matrix metalloproteinases
NF-κB	Factor nuclear kappa b
NK	Natural killer
NLRP3	NOD-like receptor protein 3
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
sIL-6R	Soluble IL-6 receptor
TGF-β	Transforming growth factor-beta
TLR-4	Toll-like receptor 4
TLRs	Toll-like receptors

TNF-α	Tumor necrosis factor-alpha
uACR	Urinary albumin creatinine ratio
UAE	Urinary albumin excretion
VCAM	Vascular cell adhesion molecule

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia, resulting from a deficiency in insulin production and/or its action. Type 2 DM is one of the most prevalent subtypes and represents an important health problem worldwide due to the epidemic proportions it has reached (Gregg et al. 2014; Cho et al. 2018). In 2019, 463 million adults between 20 and 79 years were living with DM, and projections indicate that the numbers could reach 700 million by 2045 (Saeedi et al. 2019).

Persistent hyperglycemia is associated with macro- and microvascular complications that develop over the long term in individuals with DM, which are responsible for morbidity and mortality in these patients (Sueud et al. 2019). Diabetes kidney disease (DKD) emerges as a highly prevalent microvascular complication that affects approximately 30–40% of patients with DM (Gonzalez Suarez et al. 2013). In addition to being the leading cause of end-stage kidney disease in most countries, DKD is also associated with a higher incidence of cardiovascular disease and an increased risk of mortality among diabetic individuals. Therefore, this pathology has a considerable economic and public health impact (Wada and Makino 2009), highlighting the importance of its prevention, early diagnosis, and treatment (Lee and Choi 2014).

DKD is characterized by functional, structural, and clinical renal changes caused by diabetes that persist for a period equal to or greater than 3 months, with urinary albumin excretion (UAE) $>$ 30 mg/24 h, albumin/creatinine ratio $\geq$ 30 mg/g, or glomerular filtration rate 60 mL/min/1.73m^2, after an initial phase of glomerular hyperfiltration (Mora-Fernández et al. 2014).

Currently, dysfunction in the glomerular filtration barrier that results in increased UAE is considered the first clinical sign of DKD and a predictor of disease progression (Moresco et al. 2013). However, many diabetic patients can develop DKD and even present advanced histopathological lesions when urinary albumin levels are still within the normal range (Matheson et al. 2010). These observations point to the need to study new urinary biomarkers that are more sensitive and specific than the UAE, which may allow the early detection of DKD, in addition to predicting and monitoring the progression of renal damage.

It has been recognized that tubular damage and inflammation play an important role in the pathogenesis and progression of DKD (Kim et al. 2013; García-García et al. 2014). Once activated by renal hemodynamic and metabolic changes, the inflammatory response may be present in the early stages of diabetes (Lim and Tesch 2012), resulting in increased expression of inflammatory cytokines in renal tissues, as well as increased serum and urinary levels of these molecules (Duran-Salgado and Rubio-Guerra 2014).

Clinical observations have supported the hypothesis that renal inflammation contributes to the development and progression of DKD and that urinary excretion of inflammatory markers may be useful in assessing renal damage (Wolkow et al. 2008; Cherney et al. 2012). However, studies investigating urinary levels of inflammatory cytokines in patients with DKD are still scarce. In this perspective, it is essential to evaluate the characteristics of urinary inflammatory cytokines in identifying kidney disease in diabetic patients, which may offer new opportunities for recognizing the onset, progression, and response to therapeutic interventions in DKD. These questions are essential to support the use of these markers in the assessment of kidney damage in diabetes.

Inflammation and Diabetic Kidney Disease

The pathogenesis of DKD is complex and involves many different pathways. Although metabolic derangements and hemodynamic alterations, such as hyperglycemia, oxidative stress, and renin-angiotensin-aldosterone system activation are the main factors involved in kidney damage associated with diabetes, collecting evidence call attention to the key role of systemic and local renal inflammation in the onset, development, and progression of DKD (Schena and Gesualdo 2005; Wada and Makino 2013; Tang and Yiu 2020). Several urinary and plasma inflammatory biomarkers have been reported at early and advanced stages of DKD (Navarro-González and Mora-Fernández 2008; Wada and Makino 2013). Specifically, urinary levels of interleukins are significantly increased in microalbuminuric diabetic patients with early progressive renal function decline, showing direct correlations with UAE levels and with the progression of renal impairment (Navarro et al. 2003; Nakamura et al. 2005; Wolkow et al. 2008).

Inflammation-related processes involved in the pathophysiology of DKD include the production of inflammatory cytokines, especially the interleukins and tumor necrosis factor-alpha (TNF-α); the generation of endogenous cellular adhesion molecules; the release of chemokines; and the production of growth factors (Navarro-González et al. 2011; Pérez-Morales et al. 2019). Proinflammatory cytokines, both circulating and those synthesized by immune cells in the renal tissue, can be associated with glomerular and tubular damage, oxidative stress, and fibrosis development (Neu et al. 2003; Campion et al. 2017; Perlman et al. 2015). Kidney fibrosis, which is the most critical feature of DKD, and proteinuria, an important factor in the progression of diabetic nephropathy, are both mediated through the inflammatory cells and cytokines by activating renal fibroblast cells (Kanasaki et al. 2013). The kidney inflammation and the development of fibrosis is a complex process that includes macrophages and other immune cells that release cytokines and pro-fibrotic factors (Chung and Lan 2011; Ferenbach et al. 2007) and interact with intrinsic kidney cells (Mack and Yanagita 2015) to create a pro-fibrotic microenvironment (Meng et al. 2014).

Infiltration of immune cells, mainly macrophages, is usually observed in the glomeruli and interstitium of renal biopsy samples at all stages of DKD (Klessens

et al. 2017). These cells are responsible for what is called "renal remodeling" and mainly produce numerous proinflammatory cytokines, reactive oxygen species (ROS), TGF-β, and matrix metalloproteinases (MMPs). All these factors are responsible for the local inflammation, tissue damage, and fibrosis, thus enabling the development of diabetic nephropathy (Galkina and Ley 2006). The accumulation of macrophages in the interstitium strongly correlates with renal damage in patients with DKD (Nguyen et al. 2006). Interestingly, infiltration of other immune cells, such as activated T cells, is also increased in the kidneys of patients with DKD and the number of T cells is also correlated with the degree of proteinuria (Moon et al. 2012). Studies have suggested that renal cells (including endothelial, mesangial, epithelial, tubular cells, and podocytes) and interstitial fibroblasts can also synthesize and release chemokines upon induction by growth factors and inflammatory cytokines like IL-1β and TNF-α (Panzer et al. 2006). The initial step for activation of the renal inflammatory process is not fully elucidated, but chronic exposure to hyperglycemia may be a good hypothesis.

High glucose levels induce the expression of inflammatory mediators by injured glomerular and tubular cells, contributing to morphological abnormalities and renal damage by different mechanisms: mesangial proliferation, podocyte/tubular damage, glomerular basement membrane thickness and glomerulosclerosis, and leukocyte infiltration (Pichler et al. 2017; Rayego-Mateos et al. 2020). These inflammation-induced processes occur through different signaling pathways, such as NF-kB, Janus kinase/signal transducers (JAK/STAT), TGF β, among others (Matoba et al. 2019; Alicic et al. 2017; Elsherbiny and Al-Gayyar 2016). In this context, the hyperglycemia, advanced glycation end products (AGEs), the mechanical stretch of mesangial cells, angiotensin II, ROS, and pro-inflammatory cytokines can facilitate the activation and nuclear translocation of NF-κB in renal cells (Pérez-Morales et al. 2019). Activation and nuclear translocation of NF-κB in kidneys lead to the transcription-mediated expression of multiple mediators of inflammation including chemokines, adhesion molecules, profibrotic factors, and pro-inflammatory cytokines that participate in the development and progression of DKD (Guijarro and Egido 2001; Soetikno et al. 2011).

In addition, chronic exposure to a diabetic environment can damages renal cells, resulting in injury or cell death and the release of intracellular damage-associated molecular patterns (DAMPs) into the extracellular space (Tang and Yiu 2020). DAMPs that are released in response to cell stress and injury promote inflammatory responses by binding to pattern recognition receptors (PRRs). Thus, DAMPs are recognized by PRRs such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), protease-activated receptors, the receptor for the advanced glycation end products (RAGE), lectin receptors, and other cell surface receptors (Tesch 2017). Activation of these receptors leads to inflammatory responses in renal cells. Upon binding to various PRRs, DAMPs provoke the activation of signaling cascades to secrete cytokines via the inflammasome complex (Tang and Yiu 2020).

Inflammasome acts as a complex platform to initiate a variety of cellular signaling cascades through the detection of endogenous molecules such as cytokines and

DAMPs (Ram et al. 2020). Inflammasomes can activate cytokine maturation and activation of caspases, which further leads to stimulation of the adaptive immune system (Wada and Makino 2016). Inflammasome activation can be linked to the maturation of pro-inflammatory cytokines, especially IL-1β and IL-18. Inflammasomes can also stimulate several caspase pathways leading to cell apoptosis (Martinon et al. 2002). NOD-like receptor protein 3 (NLRP3), a subfamily of NLRs, which forms an inflammasome complex by recruiting pro-caspase 1 molecule, has gained more interest in the pathophysiology of DKD (Lorenz et al. 2014; Hutton et al. 2016). The involvement of inflammatory factors in the pathogenesis of DKD is summarized in Fig. 1.

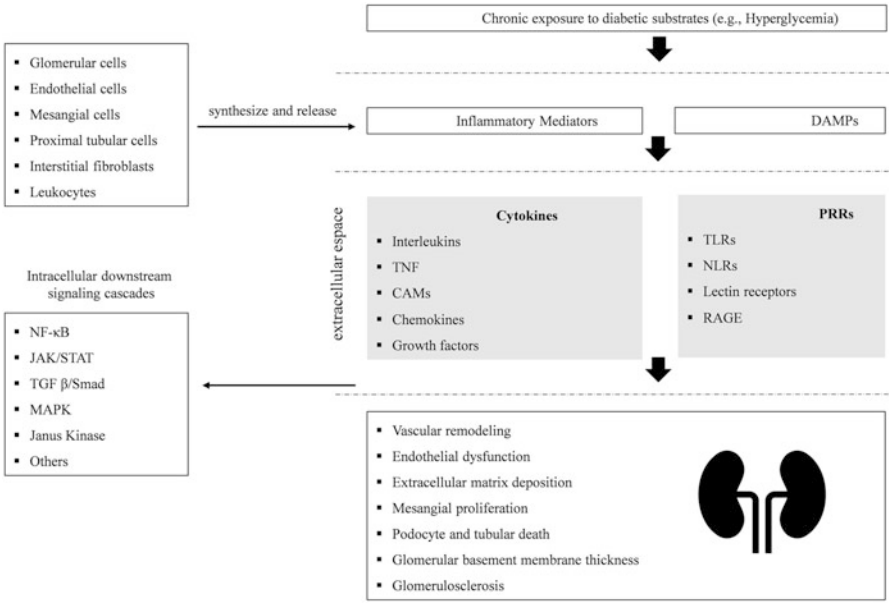

Fig. 1 Chronic exposure to the diabetic environment such as high glucose levels result in injury and the release of inflammation-related molecules. Pro-inflammatory cytokines such as IL-1, IL-6, IL-18, or TNF-α and damage-associated molecular patterns (DAMPs) were found to be involved in DKD. The DAMPs are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors and nucleotide-binding oligomerization domain-like receptors (NLRs), and others cell receptors. Activating these receptors leads to inflammatory responses (secretion of a series of pro-inflammatory cytokines and chemokines) in renal cells (such as tubular epithelial cells and mesangial cells). The signaling molecules induce the infiltration of immune cells into glomeruli and interstitium. The profibrotic effects of these cytokines are mediated by the intracellular activity of NF-κB, which is a transcription factor whose function is also associated with the progression of DKD. High levels of these cytokines have been found in the urine of patients with DKD, which has been related to increased albuminuria. In the early stages, DKD is characterized by podocytopathy and alterations to the filtration barrier, as clinically evidenced by hyperfiltration and microalbuminuria. The evolution of this disease is associated with progressive and irreversible renal fibrosis, which is orchestrated by infiltrating cells and the acquisition of profibrotic phenotypes of renal resident cells, which leads to loss of functionality and renal regenerative capacity

Urinary Interleukins

Interleukin 1

Interleukin 1 (IL-1) plays a central role in local and systemic inflammatory processes. The IL-1 cytokine family comprises 11 members: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33, and IL-1F5–IL-1F10 (Sims and Smith 2010). In this topic, actions related to proinflammatory cytokines, IL-1α and IL-1β, and an anti-inflammatory agent, the IL-1 receptor antagonist (IL-1Ra) (Rider et al. 2017; Khazim et al. 2018), will be addressed. IL-1Ra, binding to cell receptors (IL-1R), inhibits IL-1α and IL-β actions. Together, IL-1α, IL-1β, and IL-1R are crucial elements of kidney inflammation (Salti et al. 2020). The resulting biological response typically involves activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways (O'Neill 2008). Although IL-α and IL-β are simply referred to as IL-1 in many studies, they differ in several ways. While the IL-1β is secreted and circulates systemically, the IL-1α is commonly associated with the plasma membrane of the producing cell and acts locally (Dinarello 2009). In addition, IL-1β is largely produced by circulating monocytes and macrophages, whereas IL-1α is constitutively present in keratinocytes and other epithelia including tubular epithelial cells (Anders 2016).

In experimental models of DKD, Salti et al. (2020) demonstrated that hyperglycemia increases the expression of IL-1α in tubular cells, most probably a result of metabolic stress and cellular injury. Moreover, the increased renal expression of IL-1 was related to albuminuria and the accumulation of macrophages (Sassy-Prigent et al. 2000; Navarro-González et al. 2006). Lemos et al. (2018) demonstrated that IL-1β induced tubulointerstitial fibrosis throughout the activation of the MYC transcription factor, promoting deposition of fibrotic matrix. In this context, the application of a pharmacological IL-1Ra showed to prevent the progression and even reverse DKD in an animal model (Shahzad et al. 2015).

In patients with DKD, IL-1β levels were correlated with the degree of albuminuria (Jones et al. 2009). In addition, it was demonstrated that increased serum levels of IL-1α were detected in the early stage of DKD, before significant kidney impairment (Perlman et al. 2015). Notably, higher urinary concentrations of IL-1 were observed in patients with DKD than those without DKD (Sangoi et al. 2016). In addition, diabetic patients classified into microalbuminuria (uACR 30–300 mg/g creatinine) presented increased IL-1 urinary levels compared with normoalbuminuric patients (Sangoi et al. 2016).

IL-1 can alter the renal architecture, promoting the expression of chemotactic factors and adhesion molecules, increasing vascular permeability, as well as stimulating the proliferation of mesangial cells and the synthesis of extracellular matrix (Navarro-González and Mora-Fernández 2008). Specifically, IL-1 increases the production of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by different renal cells, including mesangial, endothelial, and tubular epithelial cells (Park et al. 2000), resulting in proliferation and synthesis of extracellular matrix (ECM) in the glomerular mesangium (Donate-Correa et al. 2015).

The NLRP3 inflammasome links sensing of metabolic stress in the diabetic kidney to activation of pro-inflammatory cascades via the induction of IL-1β and

IL-18 (Tang and Yiu 2020). In this context, the NLRP3 inflammasome induces the assembly of several cytosolic proteins ultimately leading to activation of caspase-1, which promotes the enzymatic activation and secretion of mature IL-1β (Anders 2016; Lei et al. 2019). Pro-IL-1β processing and IL-1β release from renal parenchymal cells have been reported (Shahzad et al. 2015). Interestingly, in those cells that coexpress IL-1α and IL-1β, IL-1α secretion can also be inflammasome-dependent, implying that IL-1α and IL-1β are released together (Yazdi and Drexler 2013). Shahzad et al. (2015) show that the levels of circulating IL-1β and IL-18 as well as renal expression of NLRP3 increased in mouse DKD models, and those increases preceded albuminuria and glomerular extracellular matrix accumulation, suggesting that inflammasome activation triggers the onset of DKD.

Interleukin 6

Interleukin 6 (IL-6) is synthesized by fibroblasts, monocytes, macrophages, T cells, and endothelial cells. Its synthesis and secretion are induced during inflammatory conditions, such as by stimulation of the TLR-4, lipopolysaccharide or cell stimulation by IL-1 or TNF-α (Kishimoto 2010). IL-6 is a multifunctional cytokine produced and expressed in renal tissue by resident (Coleman and Ruef 1992; Horii et al. 1989; Frank et al. 1993) and infiltrating (Hirano et al. 1990) cells within the kidney. Its renal expression and urinary excretion correlate with the extent of tubulointerstitial damage and mesangial proliferation (Ohta et al. 1992; Ranieri et al. 1996; Fukatsu et al. 1991; Hirano et al. 1990; Horii et al. 1989). Thus, IL-6 has direct effects on glomerular cells and infiltrated cells, inducing changes in the permeability of the glomerular endothelium, proliferation of mesangial cells, increased expression of fibronectin (Coleman and Ruef 1992), and thickening of the glomerular basement membrane (Dalla Vestra et al. 2005; Nosadini et al. 2005).

IL-6 has two different activation pathways to initiate intracellular cascades and events through a specific membrane bound receptor (mIL-6R) and a soluble form of IL-6R (sIL-6R), which are termed as classic and trans-signaling of IL-6, respectively. The classical pathway and trans-signaling of IL-6 are considered mediators of different biological processes under certain circumstances. Notably, the podocyte is the only resident glomerular cell that expresses mIL-6R and can respond to both the classical pathway and IL-6 trans-signaling (Moutabarrik et al. 1994; Coletta et al. 2000; Nechemia-Arbely et al. 2008). Classical IL-6 signaling and trans-signaling is believed to activate intracellular signaling via the gp130 cascade, for example, in kidney disorders (Scheller et al. 2011; Karkar et al. 1997). Therefore, it is speculated that the different forms of STAT3 phosphorylation are responsible for pathophysiological events distinct from classical IL-6 and trans-signaling (Lei et al. 2018).

In hyperglycemic states, both classical signaling and IL-6 trans-signaling are activated and play a detrimental role in podocyte injury (Lei et al. 2018). The podocyte is critical for glomerular filtration barrier function, and abnormalities are the major event involving the onset and progression of DKD. Kidney transplant patients with acute graft dysfunction showed specific expression of IL-6, which corresponds to the extent

of tubulointerstitial damage. Some studies have measured urine IL-6 levels in kidney transplant patients with acute graft dysfunction, yielding conflicting results (Van Oers et al. 1988; Newstead et al. 1993; Raasveld et al. 1993). Waiser and colleagues assessed urine IL-6 levels in a cohort of 145 kidney transplant patients and found the cytokine to be a sensitive indicator of rejection (Waiser et al. 1997). High serum and urinary IL-6 concentrations are associated with increased urinary albumin in patients with DKD, but serum and urinary levels do not correlate (Shikano et al. 2000; Navarro et al. 2006). Additionally, the study of Wolkow et al. (2008), which sought to verify the association of renal decline and urinary inflammatory markers in microalbuminuric patients with type 1 DM, demonstrated that urinary IL-6 concentrations are only moderately associated with decline renal function and are not related to microalbuminuria. Navarro et al. (2006) in a study carried out in a model of DM and kidney disease induction in rats demonstrated that inflammatory cytokines, including IL-6, showed a significant increase. Thus, the expression and urinary excretion of these cytokines are significantly associated with renal damage, demonstrated by the association with the values of albumin excretion in the urine. In patients with type 2 DM, urinary IL-6 can identify DKD, and its concentrations were higher among patients with normal or mildly increased Albuminuria (Sangoi et al. 2016).

Interleukin 8

Interleukin 8 (IL-8, CXCL8) is a chemotactic cytokine with a high degree of specificity for neutrophils (Mackay 2001; Gerard and Rollins 2001; Tashiro et al. 2002). This cytokine plays numerous physiological roles and can induce leukocyte activation and chemotaxis (Smith et al. 1991; Petrica et al. 2019) and lysosome release, activation, and chemotaxis (Perlman et al. 2015). In addition, it stimulates the expression of adhesion molecules by endothelial cells and antagonizes the production of immunoglobulin E (IgE) stimulated by IL-4 (Zwahlen et al. 1993). It is produced by various peripheral blood cells under a wide variety of endogenous and exogenous stimuli, especially by monocytes and macrophages and, to a lesser extent, by fibroblasts, endothelial cells, keratinocytes, hepatocytes, melanocytes, and chondrocytes (Yoshimura et al. 1987; Oppenheim et al. 1991).

Podocytes and interstitial vessel cells are the main sources of IL-8 among renal cells (Zwahlen et al. 1993). In addition, glomerular mesangial cells and renal proximal tubular cells can also produce IL-8 through pro-inflammatory stimuli, such as lipopolysaccharides, IL-1, and TNF-α (Brown et al. 1991; Gerritsma et al. 1996). IL-8 is mainly produced by the damaged glomerulus and by leukocytes infiltrating the kidneys, which may be related to the pathogenesis of interstitial inflammation and, consequently, to the acute exacerbation of kidney disease (Tashiro et al. 2002). In kidney diseases in which inflammation is involved, IL-8 expression is increased, increasing the number of endothelial cells in the inflammatory site and facilitating the recruitment and migration of leukocytes, as well as the expression of adhesion molecules (Zwahlen et al. 1993). In the diabetes scenario, it is believed that the hyperglycemic environment promotes increased serum levels of this

chemoattractant cytokine, which contributes to the initiation and progression of the inflammatory process that results in kidney damage (Feng et al. 2018).

Urinary IL-8 is increased in the early stages of DKD among normoalbuminuric patients with type 1 DM (Cherney et al. 2012) and in microalbuminuric patients with type 2 DM (Tashiro et al. 2002). In addition, there is an association between high urinary IL-8 levels and the risk of renal disease progression. Elevated urinary levels of this interleukin are associated with a decline in renal function in patients with type 1 DM (Wolkow et al. 2008). Among patients with types 1 and 2 diabetes who had kidney disease, the presence of high urinary levels of IL-8 could predict a rapid progression of the pathology (Verhave et al. 2013). For this reason, urinary IL-8 determination can be useful for both early diagnosis and monitoring of patients with DKD.

Interleukin 10

IL-10 is a pleiotropic immunoregulatory cytokine produced by Th2 cells, regulatory T cells, and monocytes/macrophages. It has anti-inflammatory properties, contributing to the regulation of the immune system by potently inhibiting the expression and/or production of inflammatory cytokines (Moore et al. 2001; Li and Flavell 2008), as well as the production of IFN-γ by T cells (D'Andrea et al. 1993). The systemic treatment with IL-10 in rats with experimentally induced glomerulonephritis significantly reduced the degree of proteinuria and systemic inflammation, attenuating kidney damage (Huang et al. 2000).

Clinical studies have found elevated serum levels of IL-10 in individuals with type 1 (Myśliwska et al. 2005) and type 2 (Wong et al. 2007; Zamauskaite et al. 1999) diabetes with DKD. Furthermore, serum IL-10 concentrations have been positively correlated with UAE (Zhang et al. 2014). In this context, Myśliwska et al. (2005) suggested that excessive production of IL-10 may indirectly contribute to DKD progression. Furthermore, Sangoi et al. (2016) demonstrated that urinary excretion of this cytokine was significantly lower in individuals with DKD than in patients without the disease, and urinary IL-10 was altered even among normoalbuminuric patients. However, studies associating IL-10 and DKD are still scarce and limited to assessing serum concentrations of this cytokine. However, it is believed that the urinary determination of this cytokine can be useful since it is altered at an early stage of DKD.

Interleukin 18

IL-18, initially identified as an interferon (IFN)γ-inducing factor, is a pro-inflammatory cytokine produced by activated macrophages and other cells. IL-18 promotes the activation of T cells and macrophages, maturation of NK cells, in addition to contributing to the production of other chemokines, promoting an increase in the synthesis of nitric oxide and the upregulation of adhesion molecules such as VCAM-1, ICAM- 1, and E-selectin in endothelial cells (Yaribeygi et al. 2019; Kaplanski 2018; Wu et al. 2008). Moreover, the pyroptotic cell death of proximal tubular cells stimulates a release of IL-18 (Miao et al. 2019).

Evidence has indicated that the inflammatory response is involved in the pathophysiology of different diseases, including diabetes, and IL-18 seems to mediate some of the processes associated with this disease since its expression is upregulated by different stimuli, including hyperglycemia (Yaribeygi et al. 2019). In a recent review, Yaribeygi et al. (2019) present some molecular pathways involving IL-18 and the development of kidney damage in diabetes. These mechanisms include the interaction of IL-18 with adhesion molecules, TNF-α, IL-1β, TGF-β, NFκb, IFN-γ, and with other processes such as apoptosis and oxidative stress (Yaribeygi et al. 2019). Thus, IL-18 plays an essential role in the early stage and progress of kidney injury in patients with diabetes and appears to play a central role as a pathogenic mediator in DKD (Yaribeygi et al. 2019; Elsherbiny and Al-Gayyar 2016).

Urine and serum IL-18 concentrations were increased in patients with type 2 diabetes and positively correlated with urinary albumin excretion rate (AER) after 6 months, supporting the strong association between IL-18 concentrations and the AER (Nakamura et al. 2005). These findings support the role of IL-18 as a key molecule in the pathophysiology of DKD. The increase in IL-18 found in patients with type 2 diabetes may occur as a consequence of the stimuli promoted by hyperglycemia, insulin resistance, and feedback mechanisms involving other cytokines (Kaplanski 2018; Yaribeygi et al. 2019).

Urinary IL-18 has also been associated with other kidney-related diseases such as nephrotic syndrome (Matsumoto and Kanmatsuse 2001), acute kidney injury (AKI) (Doi et al. 2012; Schrezenmeier et al. 2017; Miao et al. 2019), and kidney ischemia-reperfusion injury (Wu et al. 2008). The elevated urinary IL-18 appear to be more prominent in AKI than in other renal diseases, but the mechanisms involved are complex and have not yet been fully elucidated (Miao et al. 2019). Thus, evidence indicates that IL-18 may be a valuable biomarker for investigating tubulointerstitial damage, which supports its use as a diagnostic tool and a predictor of kidney disease progression.

Potential Applications to Prognosis, Other Diseases, or Conditions

Scientific evidence has shown the involvement of inflammation in the pathophysiology of kidney damage associated with diabetes. In this context, some urinary interleukins have shown potential for use as a biomarker in assessing renal injury in patients with diabetes. In addition to the role in DKD, several interleukins are involved in the pathophysiology of a range of diseases and associated complications. Concerning other kidney conditions, kidney transplant patients with acute graft dysfunction showed specific expression of IL-6, which corresponds to the extent of tubulointerstitial damage. In this context, urinary IL-6 may be a sensitive indicator of rejection. Urinary IL-18 has also been associated with other kidney-related diseases such as nephrotic syndrome, acute kidney injury (AKI), and kidney ischemia-reperfusion injury (Wu et al. 2008). The elevated urinary IL-18 appears to be more prominent in AKI than in other renal diseases, but the complex mechanisms are not yet fully elucidated (Miao et al. 2019).

Mini-dictionary of Terms

Cytokines: A group of low molecular weight polypeptides, pharmacologically active, with autocrine and paracrine effects which, in a coordinated manner, regulate inflammatory and immune responses with the participation of different cytokine-associated signaling pathways.

Damage-associated molecular patterns (DAMPs): Endogenous danger molecules that activate the innate immune system by interacting with pattern recognition receptors (PRRs). Although DAMPs contribute to the host's defense, they promote pathological inflammatory responses.

Diabetic kidney disease (DKD): A disease characterized by functional, structural, and clinical renal changes caused by diabetes that persist for a period equal to or greater than 3 months, with urinary albumin excretion (UAE) $>$ 30 mg/24 h, albumin/creatinine ratio $\geq$ 30 mg/g, or glomerular filtration rate 60 mL/min/1.73m^2, after an initial phase of glomerular hyperfiltration.

Inflammation: A process of the innate immune system activated in response to harmful conditions to maintain tissue homeostasis and integrity. However, chronic activation of the inflammatory response triggers collateral injurious effects. It is initiated when the innate immune system recognizes invading pathogens or molecules from tissue injury through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and inflammasomes of the innate immune system.

Interleukins: A group of cytokines produced by different cells in different tissues. According to their physiological actions, interleukins are classified into pro-inflammatory molecules, such as IL-1 and IL-6, or anti-inflammatory, such as IL-10.

Nuclear factor-kappa B (NF-κB): A ubiquitous transcription factor that modulates the expression of chemokines, cytokines, and adhesion molecules. This transcription factor is activated by many stimuli relevant to DKD. NF-κB can be induced in various cell types in response to many different stimuli, such as proinflammatory cytokines, oxidants, and hyperglycemia.

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs): A family of cytoplasmic pattern-recognition receptors (PRR), which play several key roles in both innate and adaptive immunity. These receptors detect intracellular pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular patterns (DAMPs).

Key Facts of Diabetes, Kidney Damage, and Urinary Interleukins

- Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia, and approximately 20–30% of patients with diabetes usually have kidney damage.
- The pathogenesis of kidney damage in DM is complex and involves various pathways, causing metabolic and hemodynamic changes.
- Systemic and local renal inflammation has crucial roles in the development and progression of kidney damage.

- Hyperglycemia leads to the expression of inflammatory mediators by injured glomerular and tubular cells, contributing to renal damage by different mechanisms.
- The renal cells (endothelial, mesangial, epithelial, tubular cells, and podocytes) can synthesize proinflammatory cytokines, which may affect different renal structures.
- Macrophages are the main inflammatory cells infiltrating the kidneys in the context of subclinical chronic inflammation associated with renal damage in DM.
- Urinary concentrations of some interleukins such as IL-1, IL-6, IL-8, and IL-18 have been associated with kidney damage among diabetic patients.

Summary Points

- Inflammatory biomarkers have been associated with kidney damage in diabetes.
- Urinary concentrations of interleukins are significantly increased in micro-albuminuric diabetic patients with early progressive renal function decline.
- Interleukins as IL-1, IL-6, and IL-18 are considered the central regulators of inflammation.
- Higher urinary concentrations of IL-1 were observed in patients with DKD.
- High urinary IL-6 concentrations are associated with increased urinary albumin excretion (UAE) in patients with DKD.
- Urinary IL-8 is increased in the early stages of DKD.
- Urinary excretion of IL-10 was significantly lower in individuals with DKD.
- Urine and serum IL-18 concentrations were increased in patients with type 2 diabetes and positively correlated with UAE.

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Serum Homocysteine as a Biomarker in Diabetes

12

Links with Variables Such as Cognition

Em Yunir and Yully Astika Nugrahayning Aziza

Contents

Introduction 281
Homocysteine and Its Role in the Body 282
Biosynthesis and Metabolism of Homocysteine 282
Factors Causing Hyperhomocysteinemia 284
Diabetes Mellitus Cellular Metabolism Disturbance and Micronutrient Status in Diabetes Mellitus 286
Hyperhomocysteinemia and Diabetes Mellitus 289
Cognitive Dysfunction in Diabetes Mellitus 289
Cognitive Function 291
Factors That Affect Cognitive Function 292
Hyperhomocysteinemia and Cognitive Function 293
Compliance in Diabetes Management and Cognitive Dysfunction 294
Applications to Prognosis 294
Mini-Dictionary of Terms 294
Key Facts of Serum Homocysteine as a Biomarker in Diabetes: Links with Variables Such as Cognition 294
Summary Points 295
References 295

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Abstract

The management of patients with type 2 diabetes mellitus (T2DM) faces its own challenges; in addition to the chronic progressive course of the disease which at some point causes various complications, it turns out that they also have to deal with behavioral conditions that hinder the management of their disease. One of the behavioral conditions encountered is related to changes in cognitive function. Many factors affect cognitive function changes in T2DM, one of which is an inflammatory condition which occurs because of hyperglycemia. Besides that, T2DM is strongly correlated with insulin resistance (IR). Inflammatory condition and IR can be caused by an increase of homocysteine (Hcy) levels. Other factors that can increase the level of homocysteine are deficiencies of vitamin B6, vitamin B12, and folic acid. Hyperhomocysteinemia (HHcy) can increase the risk of T2DM complications. This chapter will elaborate the relationship between Hcy and cognitive dysfunction in T2DM.

Keywords

T2DM · T2DM complications · Cognitive dysfunction · Hyperhomocysteine · Inflammation · Hyperglycemia · Insulin resistance · Vitamin B6 · Vitamin B12 · Folic acid

Abbreviations

3MS	Modified Mini-Mental State Exam
AGEs	Advanced glycation end products
Aβ	Amyloid beta
BBB	Blood-brain barrier
BMI	Body mass index
Cr	Chromium
CRP	C-reactive protein
Cys	Cysteine
DM	Diabetes mellitus
DN	Diabetic nephropathy
DR	Diabetic retinopathy
ER	Endoplasmic reticulum
ERKs	Extracellular signal-regulated kinases
Fe	Iron
FFA	Free fatty acid
GABA	γ-Aminobutyric acid
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
H	Hydrogen
Hcy	Homocysteine
Hcy-TL	Hcy-thiolactone
HHcy	Hyperhomocysteinemia

INSR	Insulin binding to insulin receptor
IP2	Inositol 1,3-bisphosphate
IP3	Inositol 1,4,5-trisphosphate
IR	Insulin resistance
MAPK	Mitogen-activated protein kinase
Mg	Magnesium
MMP-9	Matrix metalloproteinase-9
MMSE	Mini-Mental State Exam
MoCA	Montreal Cognitive Assessment
MTHFR	Methylenetetrahydrofolate reductase
NMDA	*N*-methyl D-aspartate
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O_2^-	Superoxide
P2X	Purinergic receptor X
P2Y	Purinergic receptor Y
PD	Parkinson disease
PKC	Protein kinase
ROS	Reactive oxygen species
RYR	Ryanodine receptor channel
S	Sulfur
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Se	Selenium
SERCA	Sarco-endoplasmic reticulum Ca2+-ATPase
SLUMS	Saint Louis University Mental Status
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
TIMP-4	Tissue inhibitor of metalloproteinase-4
UPR	Unfolded protein response
Zn	Zinc

Introduction

Hcy is an intermediate form in biochemical reaction of methionine and cysteine (Miller 2013). There are four forms of Hcy that we can find in the blood plasma, the most common form being the disulfide bound to plasma protein (mainly albumin). The main source of Hcy is dietary meal (dairy products, meats, etc.). Hcy can be converted to methionine if methionine levels are low in the body. But, when in oxidative stress condition, Hcy will be converted to cysteine which further produces glutathione as an endogenous antioxidant (Miller 2013; Hultdin 2005; Medina et al. 2001).

The normal level of Hcy is 4–10 μmol/L; when the level exceeds 15 μmol/L, the condition is called hyperhomocysteinemia (HHcy). There are several conditions that can

cause HHcy, one of which is T2DM (Miller 2013; Hultdin 2005; Medina et al. 2001). Besides that, HHcy can increase the risk of T2DM complications (Chubarov 2021; Ganguly and Alam 2015; Moretti and Caruso 2019; Tripathi 2015; Varga et al. 2005; Williams and Schalinske 2010; Zaric et al. 2019). One of the conditions that is closely related to the complications of T2DM is cognitive dysfunction. Cognitive functions that are strongly influenced by T2DM are memory function, psychomotor speed, and executive function. Cognitive dysfunction in T2DM also greatly affects compliance to the therapy, which further affects the T2DM outcomes (Moretti and Caruso 2019; Al Mutairi 2020).

Homocysteine and Its Role in the Body

Hcy is a sulfur-containing, nonproteinogenic amino acid and an intermediate form in the biochemical conversion of methionine to cysteine (Miller 2013; Hultdin 2005; Medina et al. 2001). The biochemistry form of Hcy was explained by Vincent Du Vigneaud and colleagues from the 1930s to the 1950s (Miller 2013). In 1962, Hcy was first identified in patients. These patients had homocystinuria, an inborn error of metabolism, in which homocysteine levels are very high and excreted in the kidneys in the forms of disulfide homocysteine in urine (Hultdin 2005).

The structure of Hcy and the related structure of cysteine and methionine are represented in Table 1. Hcy and cysteine have the prominent features that are free sulfhydryl groups in the end side chains of both amino acids. Sulfhydryl groups are easily to oxidation and forming disulfide linkages (Miller 2013). We can find four different forms of homocysteine in plasma: around 1% as a free thiol, 70–80% in the form of disulfide bound to plasma protein (mainly albumin), and 20–30% combines with itself to form dimer homocysteine (homocysteine) or with other thiols (Miller 2013; Tripathi 2015). Meanwhile, methionine does not have a free sulfhydryl group, so it can't form disulfide compounds (Miller 2013).

Biosynthesis and Metabolism of Homocysteine

Dietary methionine (meat, fish, dairy product, etc.) is the primary source of Hcy. Figure 1 is presenting the biosynthesis and metabolism of Hcy. Methionine is activated by addition of an adenosyl group to form S-adenosylmethionine (SAM).

Table 1 Structure and forms of homocysteine and its related amino acids

Structure of homocysteine	Forms of homocysteine in blood	
Homocysteine	Hcy-S-S-Cys-albumin	Protein-bound form
Cysteine	Hcy-S-S-Cys Hcy-S-S-Hcy	Mixed disulfide Homocysteine
Methionine	Hcy-SH	Free-reduced form

Hcy, homocysteine; S, sulfur; Cys, cysteine; H, hydrogen
Table 1 adapted from Miller JW, Encyclopedia of Human Nutrition; 2013.

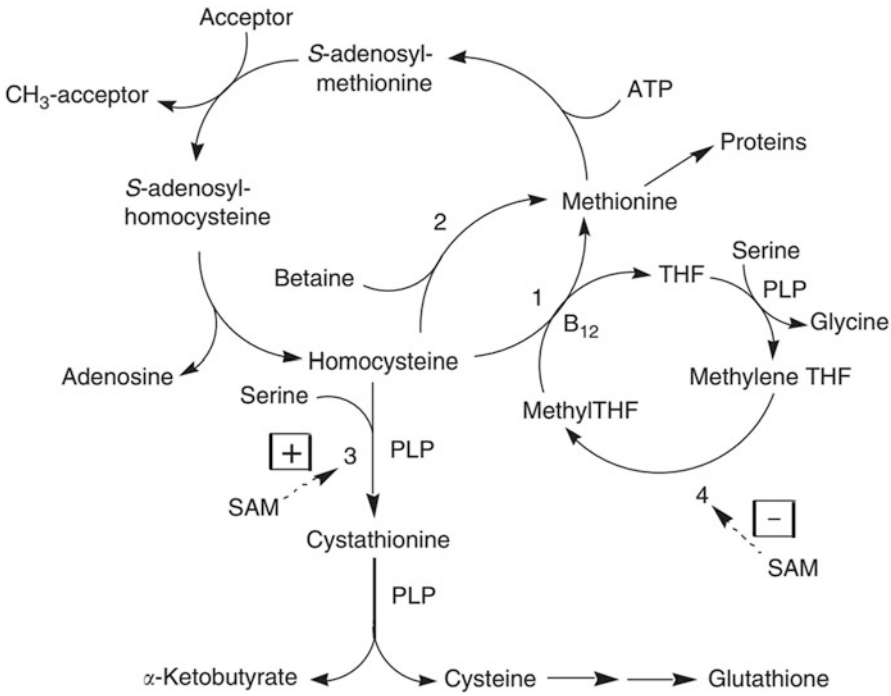

Fig. 1 The biosynthesis and metabolism of homocysteine· Key enzymes: (1) methyltetrahydrofolate-homocysteine methyltransferase or methionine synthase, (2) betaine-homocysteine methyltransferase, (3) cystathionine β-synthase, and (4) methylenetetrahydrofolate reductase (MTHFR). (Adapted from Miller JW, Encyclopedia of Human Nutrition; 2013)

SAM is a universal methyl donor in a variety essential reactions (reaction which involves DNA, RNA, protein, membrane phospholipids, neurotransmitter, and synthesis of creatine). SAM-dependent methylation reaction produces S-adenosylhomocysteine (SAH), which will be metabolized to form adenosine and Hcy. Hcy can be remethylated to form methionine or catabolized through cystathionine synthesis (Miller 2013; Hultdin 2005; Medina et al. 2001; Chubarov 2021; Ganguly and Alam 2015; Tripathi 2015; Williams and Schalinske 2010; Zaric et al. 2019).

In remethylation, Hcy regains a methyl group in a reaction that is catalyzed by methionine synthase (5-methyltetrahydrofolate-homocystein methyltransferase), with methyltetrahydrofolate as the methyl donor and vitamin B12 as a cofactor. This reaction takes place in all mammalian cells. Besides that, Hcy can be remethylated using betaine as a methyl donor and catalyzed by betaine-homocysteine methyltransferase. This reaction especially happens in the liver, kidney, and brain (Miller 2013; Hultdin 2005; Medina et al. 2001; Chubarov 2021; Ganguly and Alam 2015; Tripathi 2015; Williams and Schalinske 2010; Zaric et al. 2019).

The catabolism of homocysteine occurs through cystathionine synthesis. This reaction is catalyzed by cystathionine β-synthase which requires vitamin B6 as a cofactor. Cystathionine is broken down into α-ketobutyrate and cysteine. Furthermore, cysteine leads the formation of glutathione or inorganic sulfate (Miller 2013;

Hultdin 2005; Medina et al. 2001; Chubarov 2021; Ganguly and Alam 2015; Tripathi 2015; Williams and Schalinske 2010; Zaric et al. 2019).

Allosteric control is the important aspect in metabolism of Hcy. When dietary methionine is high, after consuming protein meal, intracellular SAM levels increase. SAM activates cystathionine β-synthase and inhibits methylenetetrahydrofolate reductase (MTHFR) and then promotes Hcy catabolism. On the other hand, when there is no dietary influx of methionine, intracellular SAM levels reduce, cystathionine β-synthase is not activated, and the inhibition of MTHFR is relived, thereby promoting Hcy remethylation (Miller 2013; Hultdin 2005; Medina et al. 2001; Chubarov 2021; Ganguly and Alam 2015; Tripathi 2015; Williams and Schalinske 2010; Zaric et al. 2019).

The level of Hcy can be controlled by oxidative stress. When the level of oxidative stress increases, Hcy will pass through toward cystathionine synthesis. So it can increase the synthesis of glutathione that serves as an important intracellular antioxidant (Miller 2013).

Hcy levels are said to be normal if they are in the range of 4–10 μmol/l. According to some literature, HHcy occurs when the level of Hcy is more than 11 μmol/l (Miller 2013), but according to some, HHcy occurs when the level of Hcy is more than 15 μmol/l (Tripathi 2015). Here, the HHcy categories will be explained: mild-moderate HHcy in the range 11–25 μmol/l or 16–30 μmol/l; intermediate HHcy in the range 26–50 μmol/l or 31–100; and severe HHcy in the range more than 50 μmol/l or more than 100 μmol/l (Miller 2013; Tripathi 2015).

Factors Causing Hyperhomocysteinemia

Hcy levels can be increased due to several conditions such as deficiency of B vitamins and folate in the diet, all diseases that disrupt the absorption of B12 or folate (atrophic gastritis, after gastrectomy, pancreatic disease, malabsorption in the small intestine, celiac disease, etc.), chronic kidney disease, liver disease, low levels of thyroid hormones (hypothyroidism), psoriasis, certain medications (such as antiepileptic drugs and methotrexate), genetic mutation of MTHFR, systemic lupus erythematosus, smoking, heavy alcohol consumption, sedentary lifestyle, etc. (Miller 2013; Hultdin 2005; Moretti and Caruso 2019; Varga et al. 2005; Al Mutairi 2020; Škovierová et al. 2016).

Vitamin B12 has a role as a cofactor in remethylation (recycling homocysteine to methionine); meanwhile, vitamin B6 has a role as a cofactor in catabolism of homocysteine (transsulfuration) (Miller 2013; Hultdin 2005; Al Mutairi 2020). Deficiency in folic acid, vitamin B12, and vitamin B6 can cause HHcy. Deficiency in vitamin B6 only causes prandial state plasma Hcy increase or slightly increased fasting Hcy level; meanwhile, deficiency in folic acid and vitamin B12 can elevate prandial and fasting Hcy level (Hultdin 2005).

HHcy can cause biotoxicity by several mechanisms: (a) modification of protein structure (homocysteinylation), (b) induction of oxidative stress, and (c) excitotoxicity (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019).

(a) Homocysteinylation

The degree of homocysteinylation is proportional to the level of Hcy. *N*-homocysteinylation formed when Hcy interacts by its amino group with ε-amino group of lysine residue in protein. This is a result of high reactivity of Hcy-thiolactone (Hcy-TL) (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019). *N*-homocysteinylation disrupts protein function through the introduction of new free thiol groups and inactivation of free amino group; it affects the overall redox potential and increases oxidative stress. The pathological consequences are cytotoxicity through endoplasmic reticulum (ER) stress, activation of unfolded protein response, enhanced protein degradation, enzymatic inactivation, and amyloid formation. Besides that, it can act as neoantigens which can trigger the activation of the inflammatory response as a key component of atherogenesis, atherothrombosis, and stroke etiology. *N*-homocysteinylation in the luminal face of vascular endothelial cells promotes neoantigen-autoantibody interaction which can lead to the activation of circulating macrophages and cause repeated vascular endothelium damage. Furthermore, Hcy-TL disrupts the regeneration of vascular endothelium itself by direct inhibition of lysyl oxidase, which has a strong impact on the vascular stiffness (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019).

(b) Induction of Oxidative Stress

HHcy can increase the production of reactive oxygen species (ROS) by several mechanisms: (i) autoxidation, (ii) inhibition of the enzymatic activity of antioxidant in cells, (iii) disruption of extracellular superoxide dismutase from endothelial surfaces, (iv) activation of NADPH oxidases, and (v) nitric oxide synthase (NOS)-dependent generation of superoxide anion. ROS and oxidative stress form the nitrotyrosine which lead the formation of the strong oxidant peroxynitrite. Peroxynitrite causes the alteration of protein function and induces cellular dysfunction (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019).

(c) Excitotoxicity

HHcy could lead to neuronal cell death due to transient activation of extracellular signal-regulated kinases (ERKs), mitogen-activated protein kinase (MAPK), and p38 MAPK. HHcy can cause intracellular Ca^{2+} mobilization and ER stress which is followed by apoptotic events, remodeling of extracellular matrix in brain parenchyma, and endothelial dysfunction (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019).

HHcy itself is able to induce disruption of blood-brain barrier (BBB), by several methods: (i) HHcy induces an imbalance activity between matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-4 (TIMP-4); (ii) HHcy acts as an excitatory neurotransmitter for γ-aminobutyric acid (GABA) receptors which increased permeability of vascular and *N*-methyl D-aspartate (NMDA) receptor which increased cell susceptibility to excitatory amino acids (Škovierová et al. 2016; Brustolin et al. 2010; Kim et al. 2018; Kluijtmans et al. 2003; Tinelli et al. 2019).

Diabetes Mellitus Cellular Metabolism Disturbance and Micronutrient Status in Diabetes Mellitus

T2DM is a chronic lifestyle disease, the most common noncommunicable disease. T2DM is characterized by relative deficiency of insulin and insulin resistance (IR) in target tissues. Besides that, there are some conditions that perpetuate T2DM (Galicia-Garcia et al. 2020; Hameed et al. 2015; Holt et al. 2017; Maciejczyk et al. 2019; Nolan and Prentki 2019; Prentki et al. 2020). Here, we will discuss all of the processes:

1. β-Cell Pancreas Dysfunction

   β-cell pancreas is the target organ in stress condition (inflammation, ER stress, metabolic/oxidative stress, etc.) that can cause disruption in the β-cell pancreas integrity. Some conditions, such as excess of free fatty acid (FFA), hyperglycemia, lipotoxicity, glucotoxicity, and glucolipotoxicity, can activate pro-apoptotic signal which can cause inflammation and disrupt the integrity of β-cell pancreas. Further, this condition can intrude insulin secretion and chronic hyperglycemia condition which leads to T2DM (Galicia-Garcia et al. 2020; Hameed et al. 2015; Nolan and Prentki 2019; Prentki et al. 2020).
2. Some Conditions Perpetuating T2DM
   (a) Nutrition

   High-calorie food can increase the ROS which leads to inflammatory condition. Pro-inflammatory condition also can cause mitochondrial dysfunction, ER stress, activate NADPH oxidase (NOX), and superoxide (O_2^-) production (Galicia-Garcia et al. 2020; Hameed et al. 2015; Holt et al. 2017; Maciejczyk et al. 2019; Nolan and Prentki 2019; Prentki et al. 2020). Activation O_2^- of can activate five major pathways in T2DM, that is (Galicia-Garcia et al. 2020; Hameed et al. 2015):
   - Polyol pathway
   - Increase the formation of advanced glycation end products (AGEs)
   - Increase the expression of AGE receptor and its activating ligands
   - Activation of protein kinase (PKC) isoform
   - Overactivity of hexosamine pathway

   (b) Sedentary lifestyle

   Sedentary lifestyle can cause obesity which can lead to chronic low-grade systemic inflammation and T2DM (Galicia-Garcia et al. 2020).

   (c) Gut dysbiosis

   Gut microbiota play an action to produce metabolite. When gut dysbiosis happens, it can disrupt gut barrier, proliferation of β-cell pancreas, and biosynthesis of insulin, so it can lead to IR and T2DM (Galicia-Garcia et al. 2020).

   (d) Metabolic memory

   Metabolic memory happens though four mechanisms: epigenetics, oxidative stress, nonenzymatic glycation of protein, and chronic inflammation. Metabolic memory happens in T2DM, so it can lead to T2DM complication (Galicia-Garcia et al. 2020).

(e) Mitochondrial dysfunction

Mitochondrial dysfunction happens because of chronic inflammation. Mitochondrial dysfunction has bidirectional relationship with T2DM. It can cause the T2DM itself, or on the other hand it can happen because of T2DM (Galicia-Garcia et al. 2020; Hameed et al. 2015).

3. IR

Insulin action in fed state is influenced by insulin growth factor-1 (IGF-1). Meanwhile in fasting condition, the insulin action is restricted by counter-regulatory hormone (glucagon, glucocorticoid, and catecholamine). IR in the target organ is major contributor in T2DM pathogenesis. There are three target organs that are closely related to IR (Galicia-Garcia et al. 2020). The three organs will be described in the following:

(a) Skeletal muscle

Insulin in the skeletal muscle will be binding with insulin receptor (INSR) and cause translocation of glucose transporter 4 (GLUT4) to the plasma membrane, then increase the glucose uptake from plasma and cause the glycogen synthesis. IR in the skeletal muscle is caused by sedentary lifestyle and mutation in insulin receptor in the target organ (Galicia-Garcia et al. 2020).

Sedentary lifestyle leads to obesity. Obesity causes increasing immune cell infiltration and secretion of pro-inflammatory molecules in intermyocellular and perimuscular adipose tissue that leads to skeletal muscle inflammation and IR through paracrine effect (Galicia-Garcia et al. 2020).

(b) Adipose tissue

Insulin has a role in stimulating glucose uptake, synthesis triglyceride, suppressing triglyceride hydrolysis, and uptake of FFA and glycerol from circulation. High-fat diet can cause adipocyte hypertrophy which increases pro-inflammatory cytokines and metabolic inflammation, so causing IR and T2DM. When IR happens, it can suppress the lipolysis, disrupt glucose uptake, and increase the FFA to the circulation, thereby exacerbating hyperglycemia (Galicia-Garcia et al. 2020).

(c) Liver

High-fat diet will interfere insulin signaling in the liver which can disrupt glycogen synthesis, increase gluconeogenesis, trigger lipolysis, and increase inflammatory factors such as C-reactive protein (CRP). All of these conditions cause hyperglycemia and inflammation that trigger T2DM (Galicia-Garcia et al. 2020).

(d) IR in the Brain

IR in the brain can cause overproduction of ROS, so it can increase permeability of membrane cell, ATP depletion, and accumulation of protein aggregates. This condition can change the neurite overgrowth, disruption in neuroplasticity, and disruption in uptake and release of neurotransmitters. Besides that, IR in peripheral can decrease the number of insulin receptors in endothelial and increase the permeability of BBB to several substances. All of these conditions can induce cognitive dysfunction in T2DM (Maciejczyk et al. 2019).

In vitro young-aged models show that directly administered insulin into the brain can improve learning and memory. This result shows that insulin has important role for cognitive function (Adzovic et al. 2015).

Micronutrient is an important component to support metabolic cell activity. In glucose metabolism, micronutrient is needed to activate insulin receptor. Chronic hyperglycemia can change micronutrient status in the body and cause stress oxidative condition which further induces IR, T2DM, and its complications. The following will explain the status of micronutrients and their impact on the condition of T2DM patients (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(a) Zinc (Zn)

Zinc is abundant in the pancreas, and the amount is tightly regulated. Zn dysregulation causes disturbances in insulin synthesis, storage, and secretion as well as glycemic control. In T2DM patients, the level of Zn in the blood plasma has decreased, while the excretion of Zn in the urine has increased (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(b) Magnesium (Mg)

Magnesium (Mg) is an important component to increase insulin's ability to activate tyrosine kinase for glucose metabolism. Low magnesium levels can be a consequence of chronic hyperglycemia or a cause of IR. The cause of low Mg levels in T2DM patients is still not known. There are several hypotheses of low Mg levels in T2DM patients, such as the following: the condition of IR triggers an increase in the excretion of Mg in the urine, so that the Mg level in the plasma becomes low (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(c) Chromium (Cr)

Chromium (Cr) levels in T2DM patients decreased by about 30%, and its excretion in the urine increased to 100% due to increased glomerular hyperfiltration (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(d) Iron (Fe)

Iron (Fe) is a strong pro-oxidant that can increase ROS production, thereby increasing oxidative stress. In T2DM with poor glycemic control, there is an increase in Fe levels, so that oxidative stress condition increases (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(e) Selenium (Se)

Selenium (Se) acts as insulin-like actions that function to regulate specific genes in pancreatic cells and improve islet function. In T2DM patients, Se levels decrease with a mechanism that is unknown (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

(f) Vitamins

The B group of vitamins plays an important role in glucose metabolism. B vitamins are water soluble, so that in T2DM patients with poor glycemic control, there can be an increase in the excretion of B vitamins (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

Vitamin A acts as a diphasic concentration-dependent effect on insulin release. Meanwhile, in T2DM patients, the levels have decreased by a mechanism that is not known (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

Vitamin C is required for collagen synthesis and is an important antioxidant that the body can't produce. In T2DM patients, vitamin C levels have decreased due to increased excretion and the high need to overcome oxidative stress conditions (Kaur and Henry 2014; Valdés-Ramos et al. 2015).

Vitamin D has important role in the body. It regulates the secretion of adipokine hormone which maintains the homeostasis of lipid and glucose metabolism. Vitamin D also increases the expression of insulin receptors. When deficiency vitamin D occurs, it can decrease the expression of insulin receptors. Besides that, vitamin D maintains the mitochondrial activity to produce antioxidant endogens, so it can decrease the level of ROS. When there is deficiency in vitamin D, the level of ROS will be increase which leads to inflammatory condition and increases the level of Ca^{2+}. Overexpression of Ca^{2+} can induce apoptosis and cell death of the pancreatic β-cell. This condition can cause decreasing the secretion of insulin and cause diabetes mellitus (Berridge 2017; Kostoglou-Athanassiou et al. 2013; Martin and Campbell 2011; Wiernsperger and Rapin 2010).

Hyperhomocysteinemia and Diabetes Mellitus

HHcy is an important biomarker to increasing the risk of type 2 diabetes mellitus (T2DM). Besides that, HHcy can increase the complication of T2DM. The level of Hcy can be affected by several factors, such as age, sex, genetics, duration of T2DM, glucose level, body mass index (BMI), dietary habit, therapeutic schedule, renal function, and metabolism pathway of Hcy (Platt et al. 2017; Wang et al. 2021). The main mechanisms that affected Hcy level in T2DM are as follows: (1) the resistance of insulin, besides that HHcy also contributes to the development of IR. Hcy also acts as neurotoxins. The structure of pancreatic β-cell has some similarities with the structure of neuronal cells, so it can cause dysfunction of insulin secretion. The main characteristic of T2DM is IR and insufficiencies of insulin secretion (2). HHcy promotes vascular endothelial injury and microvascular damage, so it can cause disruption in microcirculation and microthrombus in the kidney, liver, etc. Increasing of Hcy levels in blood has a potential risk factor for the development of diabetic nephropathy (DN) and diabetic retinopathy (DR) (Platt et al. 2017; Wang et al. 2021).

Cognitive Dysfunction in Diabetes Mellitus

Cognitive dysfunction occurs in 19.5–21.8% of patients with T2DM. This condition can happen because of several mechanisms (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).

1. Hyperglycemia

   Hyperglycemia can decrease cognitive function through polyol pathway, increasing the formation of AGEs and diacylglycerol activation of protein kinase C and increasing glucose shunting in the hexosamine pathway. Long-term hyperglycemia can increase cerebral muscle basement membrane and so decrease the cerebral blood flow circulation and directly damage neurons. Reduced cerebral blood flow can disrupt learning and memory ability. Besides that, hyperglycemia can disrupt fibronectin (glycoprotein macromolecular in plasma and extracellular matrix) which further causes proliferation of capillaries in the brain, increases the permeability of BBB, and increases influx of inflammatory cells antibodies which attack the brain cells and cause decrease in cognitive function (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).
2. Vascular disease

   T2DM has two to six times increased risk of thrombosis. Besides that, it can cause accumulation of glutamate and lactate and decreasing cerebral blood flow. This condition increases the risk of ischemia, which leads to cognitive dysfunction (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).
3. Hypoglycemia

   Long-term severe hypoglycemia can cause multifocal/diffuse necrosis of cerebral cortex and chromogenesis of ganglion cells which can cause cognitive dysfunction (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).
4. IR and amyloid deposition

   IR or insulin deficiency can induce impairment in glucose metabolism and brain energy balance, which can increase oxidative stress, production of ROS, deoxyribonucleic acid damage, and mitochondrial dysfunction. All of these conditions can induce pro-apoptosis, pro-inflammatory, and pro amyloid beta (Aβ) cascade which further lead to cognitive dysfunction (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).

Furthermore, T2DM has a correlation with the disruption of lipid metabolism, leading to the increase of total cholesterol (TC) and triglyceride (TG), which can cause endothelial dysfunction and accumulation of Aβ protein and further cause cognitive dysfunction. Increasing of TC can cause hippocampal synaptic transmission which can impair memory formation (Kim 2019; Kodl and Seaquist 2008; Sun et al. 2020).

Cognitive functions that are mainly affected by T2DM are memory function, psychomotor speed, and executive function (Munshi 2017).

(a) Impairment in memory function can cause several impacts in diabetic patients such as forgetting to monitor blood glucose, to take medication, to take a meal, to attend consultations, etc. These problems can be overcome by several strategies such as decreased frequency of self-monitoring and asking caregivers to help; giving pillboxes, alarm, and long-acting formulation therapy; reminders to clinic visit; etc. (Munshi 2017).

(b) Impairment in executive function such as difficulty in problem-solving, difficulty stopping old behavior and starting new behavior, difficulty with mental flexibility, etc. These problems can be overcome by several strategies such as repeating the instruction and education, avoiding labeling such as "noncompliant," giving simple regimen, maintaining the old therapy if possible, asking caregivers to help, simplifying the instruction and regimen therapy, etc. (Munshi 2017).

Cognitive Function

Cognitive function is a term that refers to mental processes which are involved in the acquisition of knowledge, manipulation of information, and reasoning (Miller 2013; Hultdin 2005; Medina et al. 2001; Tripathi 2015). This function is divided into five domains, that is, attention, memory, visuospatial, language, and executive function. All of the domains cannot stand by themselves; they are interconnected with each other (Mayza and Lastri 2017). The impairment of cognitive function happens because of damage of the structure or function of the brain. It can happen because of several factors, i.e., age and some diseases such as hypertension, diabetes, dyslipidemia, nutritional disorders, vascular diseases (cerebrovascular, heart, kidney, etc.), and autoimmune disease (Valdés-Ramos et al. 2015).

There is no single ideal tool that is recommended for screening cognitive function (Hultdin 2005; Medina et al. 2001). A variety of cognitive screening tools are available for bedside/clinical assessment (Mayza and Lastri 2017). Every screening tool has its own sensitivity and specificity. Comprehensive screening test should include five domains of cognitive function. Some of screening tools will be explained below (Segal-Gidan 2013).

(a) Mini-Mental State Exam (MMSE)

MMSE has sensitivity of 69–91% and specificity of 87–99% and consists of 19 items with a maximal score of 30. Cognitive functions assessed include orientation, registration, attention, and calculation; short-term verbal recall; naming; repetition; three-step command; reading; writing; and visuospatial (Segal-Gidan 2013).

(b) Modified Mini-Mental State Exam (3MS)

3MS has sensitivity of 83–94% and specificity 85–90% and consists of 15 items with a maximal score of 100. Cognitive functions assessed include orientation, registration, attention, and calculation; short-term verbal recall; delayed recall; category fluency, executive function, and naming; repetition; three-step command; reading; writing; and visuospatial (Segal-Gidan 2013).

(c) Mini-Cog

Mini-Cog has sensitivity of 76–99% with specificity of 89–93%. It consists of two items with a maximal score of 5. Cognitive functions assessed include visuospatial, executive functioning, and short-term recall and include clock drawing (Segal-Gidan 2013).

(d) Montreal Cognitive Assessment (MoCA)
MoCA has sensitivity of 100% and specificity of 87%. It consists of 12 items with a maximal score of 30. Cognitive functions assessed include visuospatial/executive functioning, naming, attention, repetition, verbal fluency, abstraction, short-term verbal recall, and orientation and include clock drawing (Segal-Gidan 2013).

(e) Saint Louis University Mental Status (SLUMS)
SLUMS has sensitivity of 92–95% and specificity of 76–81%. It consists of 11 items with a maximal score of 30. Cognitive functions assessed include orientation, verbal recall, calculation, naming, attention, and executive function and include clock drawing (Segal-Gidan 2013).

(f) Clock Drawing Test
Clock drawing test has sensitivity of 88% and specificity of 71%. It consists of one item with a score of 4–10. Cognitive functions assessed include visuospatial and executive function (Segal-Gidan 2013).

Factors That Affect Cognitive Function

There are some factors that affect cognitive function, such as age, level of education, sex, physical activity, cigarettes, alcohol, depression, social factors like social activity and occupation, medical history/diseases, and BMI. Some diseases have a correlation with cognitive function such as hypertension, stroke, T2DM, and metabolic syndrome (Adamowicz et al. 2020; Brant et al. 2018; Chen and Chang 2016; De Silva and Faraci 2016; Rensma et al. 2020). Hypertension correlates with endothelial dysfunction so it causes disruption in BBB permeability, inward vascular remodeling, vascular remodeling, and rarefaction, which further causes the cognitive dysfunction (Adamowicz et al. 2020; Brant et al. 2018; Chen and Chang 2016; De Silva and Faraci 2016; Rensma et al. 2020). Stroke can occur due to bleeding or thrombosis, where this condition will cause impaired perfusion in certain regions of the brain, so that it can affect cognitive function (Adamowicz et al. 2020; Brant et al. 2018; Chen and Chang 2016; De Silva and Faraci 2016; Rensma et al. 2020).

T2DM causes structural and functional change in the brain. Structural changes that occur include collagen deposition, thickening of the basement membrane, reduced microvascular density, and loss of collateral vessels, while functional changes occur due to endothelial dysfunction (endothelium-dependent vasodilation, enhanced thrombosis, loss of BBB integrity) causing changes in myogenic response, response to increase or decrease of pressure in brain microvascular, and cognitive dysfunction. In addition, there are several conditions that correlate with DM such as IR, glucose toxicity, and inflammation. This condition causes cognitive dysfunction, as can be explained as follows (Adamowicz et al. 2020; Brant et al. 2018; Chen and Chang 2016; De Silva and Faraci 2016; Rensma et al. 2020):

- IR affects the coordination of nerve in the brain and reduces or blocks blood flow to the brain.
- Glucose toxicity causes vascular damage and reduces nerve regeneration, so it can disrupt cognitive function like memory function and attention

Metabolic syndrome correlates with low-grade chronic systemic inflammation and is associated with poor collateral supply in the pial circulation and microcirculation in leptomeninges (Adamowicz et al. 2020; Kim and Park 2017; Pengpid et al. 2019).

Hyperhomocysteinemia and Cognitive Function

Hcy acts as a neurotransmitter; it has a role as an excitatory agonist on the NMDA receptor subtype of glutamate receptors and is also involved in NMDA modulatory site. Hcy-NMDA binding can enhance calcium influx but depends on glycine concentration. In the normal level of glycine and Hcy does not cause toxicity. Meanwhile, when the level of glycine is normal but there is a HHcy, it can cause neurotoxicity. In the case of head trauma or stroke, the glycine levels will increase which can cause neurotoxic effect and enhance calcium influx; even the Hcy level is low (Ganguly and Alam 2015; Moretti and Caruso 2019).

HHcy contributed to cognitive decline, white matter damage, brain atrophy, neurofibrillary tangles as a tau protein accumulation in Alzheimer's disease, and dementia. Giving Hcy in animal models can increase the formation of β-amyloid plaque, the process similar to the onset of Alzheimer's disease. The elevated blood levels of homocysteine also contributed to the changes of integrity and function of BBB. People with hypertension mostly have a high level of homocysteine, which can increase the risk of stroke. In people with stroke, blood supply to the brain is interrupted, so resulting in neuronal cell death and loss of function in the specific brain regions (Miller 2013; Damanik et al. 2019; Kumar et al. 2017; Price et al. 2018; Smith and Refsum 2016). Besides that, HHcy causes cognitive decline and depression in patients with Parkinson disease (PD). In PD cases, Hcy mediated oxidative stress/damage in dopaminergic neurons of substantia nigra, the hallmark of the disease (Miller 2013; Damanik et al. 2019; Kumar et al. 2017; Price et al. 2018; Smith and Refsum 2016).

We can conclude that HHcy is associated with cerebrovascular disease and stroke, as previously explained that these conditions cause cognitive dysfunction. Cerebral microcirculation is very sensitive to local homocysteine levels. Increased levels of homocysteine can cause endothelial dysfunction, increase oxidative stress, and increase expression of matrix metalloproteinases, causing basement membrane irregularities, degeneration of pericyte, disruption of the BBB, and ultrastructural changes in the endothelial mitochondria (De Silva and Faraci 2016). HHcy strongly correlated with cognitive dysfunction. HHcy is also present in T2DM. So people with T2DM and HHcy have poorer cognitive function (Robbins et al. 2005).

Compliance in Diabetes Management and Cognitive Dysfunction

T2DM has a strong association with cognitive dysfunction. But HHcy is not strongly correlated with cognitive dysfunction in global condition (Damanik et al. 2019). Cognitive function is an independent factor in compliance with the treatment in T2DM (Świątoniowska-Lonc et al. 2021). This hypothesis supported by meta-analysis states that there is no association between mild cognitive dysfunction and compliance to antidiabetic therapy (Mirghani et al. 2021). Meanwhile, some literature concludes that cognitive function and psychological condition contribute to adherence of treatment in T2DM. Psychological condition such as depression can cause nonadherence to T2DM treatment, because of patient's lack of networking and social support. Cognitive dysfunction such as lack of executive function, memory, learning, attention, etc., causes difficulty in T2DM patients in adapting to complex therapeutic regimens or new therapy. Uncontrolled T2DM can increase the morbidity and mortality (Mendes et al. 2019).

Applications to Prognosis

Hcy is one of the important amino acids in the body. Hcy can be directed toward catabolic reactions to cysteine when oxidative stress is increased. This reaction is intended to produce glutathione as an endogenous antioxidant. However, if HHcy occurs, it will cause an increase in ROS production which will trigger inflammatory conditions. HHcy is strongly correlated with T2DM. HHcy can act as a risk factor for T2DM, but on the other hand HHcy can also worsen complications in T2DM. One of the complications of T2DM that occurs is cognitive dysfunction. Cognitive dysfunction can affect the compliance of T2DM management. If all of these conditions are not well controlled, it will increase the morbidity and mortality in T2DM.

Mini-Dictionary of Terms

- Cognitive dysfunction: Condition where there is impairment in one of cognitive domains
- Homocysteine: Intermediate protein between methionine and cysteine reaction
- IR: Condition when the target tissues do not respond anymore to insulin
- T2DM: Disease that is caused by IR and inflammatory condition as the effect of long-term hyperglycemia

Key Facts of Serum Homocysteine as a Biomarker in Diabetes: Links with Variables Such as Cognition

- Uncontrolled T2DM can cause several complications, such as cognitive dysfunction.

- Chronic complication occurs because of long-term inflammation and IR.
- Cognitive dysfunction occurs in 19.5–21.8% of patients with T2DM.
- Main cognitive dysfunctions that are affected in T2DM are memory function, psychomotor speed, and executive function.
- So many factors can cause inflammation; one of the factors is hyperhomocysteinemia.

Summary Points

- Metabolism changes in the brain occur because of chronic inflammation and IR.
- Hyperhomocysteinemia is one of sources of inflammation.
- Cognitive dysfunction can affect the compliance of T2DM management.
- Poor compliance inT2DM can affect the target therapy.
- Poor glycemic control can increase the mortality and morbidity.

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3-Hydroxyisobutyrate (3-HIB): Features and Links as a Biological Marker in Diabetes

13

Simon Nitter Dankel

Contents

Introduction 300
BCAA Catabolism in Adipose Tissue 301
3-HIB in Interorgan Metabolic Crosstalk 302
3-HIB Reflects Hepatic Fatty Acid Oxidation 303
Conclusion and Future Perspectives 304
Applications to Prognosis, Other Diseases, or Conditions 304
Mini-Dictionary of Terms 305
Key Facts of Diabetes 305
Summary Points 306
References 307

Abstract

Obesity and type 2 diabetes increase morbidity and mortality via altered energy metabolism, associated with altered circulatory concentrations of amino acids and lipids as well as glucose. Concentrations of 3-hydroxyisobutyrate (3-HIB), a specific intermediary metabolite in the degradation of the branched-chain amino acid (BCAA) valine, show a strong and stepwise increase in the circulation of people with prediabetes and type 2 diabetes, at least in part reflecting progression of systemic insulin resistance. Conversely, transient increases in circulatory 3-HIB concentrations during fasting are seen in healthy people, likely as a mechanism that normally contributes to cellular adaptations to fluctuating food intake. However, chronic changes in BCAA metabolism and elevations of 3-HIB may reflect a chronic perturbation in energy metabolism that underlies diet-induced pathogenic insulin resistance and type 2 diabetes.

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Keywords

Branched-chain amino acids · Adipose tissue · Liver · Skeletal muscle · Metabolism · Lipid storage · Mitochondria · Fatty acid uptake · Obesity · Type 2 diabetes

Abbreviations

2AAA	2-Aminoadipic acid
3-HIB	3-Hydroxyisobutyrate
BCAA	Branched-chain amino acid
CoA	Coenzyme A
HIBCH	3-Hydroxyisobutyryl-CoA hydrolase
MMA	Methylmalonic acid
Pgc-1α	Peroxisome proliferator-activated receptor-γ coactivator 1 alpha
ROS	Reactive oxygen species
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TTA	3-thia fatty acids

Introduction

Overweight and obesity raise the risk of hyperglycemia, a particularly strong risk factor linked to type 2 diabetes as well as many other common diseases, including cardiovascular diseases (CVD), dementia, nonalcoholic fatty liver disease, and several cancers. Obesity promotes type 2 diabetes and the risk of other diseases in large part via increased accumulation of fat in the intra-abdominal region (visceral adiposity) and in ectopic sites such as the liver, pancreas, and muscles, in close association with glucose intolerance, insulin resistance, dyslipidemia, high blood pressure, and arterial stiffness (Haslam and James 2005; Van Gaal et al. 2006). Type 2 diabetes now affects more than 450 million people worldwide, nearly 10% of the world's population, and is projected to affect 700 million people by 2045 (Saeedi et al. 2019). The primary diagnostic criterion for type 2 diabetes is glycated hemoglobin (HbA1c) in the circulation of at least 48 mmol/mol (6.5%). An even greater number of people may have HbA1c in the prediabetes range (between 6 and 6.5%). Dietary or other interventions that reduce obesity and ectopic fat accumulation can lower HbA1c in patients with type 2 diabetes, allowing remission of the disease (Taylor et al. 2021). However, there is considerable individual heterogeneity in the pathogenic pathways of type 2 diabetes (Ahlqvist et al. 2020), and biological variability in response to interventions such as diet (Lean et al. 2018) and exercise (Solomon 2018), underscoring the need to identify early markers of individual disease pathways as well as indicators of appropriate individual treatments.

In recent years, the important role of altered amino acid metabolism in obesity, insulin resistance, type 2 diabetes, and other lifestyle-related conditions has become increasingly clear. Cohort studies comparing people with and without obesity,

insulin resistance, or type 2 diabetes have shown circulatory elevations particularly of branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) and glutamate in these conditions, and reductions particularly in glycine (Newgard et al. 2009; Yamakado et al. 2012; McCormack et al. 2013; Batch et al. 2013; Badoud et al. 2014; Thalacker-Mercer et al. 2014; Lynch and Adams 2014; Takashina et al. 2016). Data from the cross-sectional METSIM study for 4,851 men with 7.4-year follow-up showed significant independent associations between incident type 2 diabetes and elevated circulating BCAAs and glutamate as well as alanine, tyrosine, and aspartate, linked not only to insulin resistance but also to insulin secretion (Vangipurapu et al. 2019). In a 10-year follow-up cohort of 2,519 patients with coronary heart disease and no diabetes at baseline, independent associations have additionally been observed for plasma arginine and asparagine with incident diabetes (McCann et al. 2019).

Metabolomics profiling has also revealed altered circulatory concentrations of specific intermediates of amino acid metabolism in people with obesity, insulin resistance, and/or incident type 2 diabetes, such as alpha-hydroxybutyrate (Gall et al. 2010; Ferrannini et al. 2013; Peddinti et al. 2017), 2-aminoadipic acid (2AAA) (Wang et al. 2013; Lee et al. 2019), and 3-hydroxyisobutyrate (3-HIB) (Mardinoglu et al. 2018; Andersson-Hall et al. 2018; Nilsen et al. 2020). These metabolites are formed in distinct amino acid pathways and may therefore more directly reflect specific underlying pathogenic mechanisms than the amino acids they are derived from. Moreover, such metabolites can also play an active role in metabolic homeostasis by directly modulating metabolic processes in target cells, as shown for 2AAA (Xu et al. 2019) and 3-HIB (Jang et al. 2016; Nilsen et al. 2020).

Generally, maintained activity of the early rate-limiting step in BCAA catabolism, at the level of the irreversible decarboxylation of branched-chain α-keto acids (BCKAs) catalyzed by BCKA dehydrogenase (BCKD), was recently shown to ameliorate insulin resistance in mice, with supportive causal evidence also in humans (Zhou et al. 2019). These observations highlight the therapeutic potential of targeting BCAA catabolism. However, deeper knowledge of tissue-specific alterations in BCAA metabolism in obesity and insulin resistance is needed to identify more specific targets to fully harness this potential. A mouse study using isotopic tracing revealed particularly high BCAA oxidation to fuel the tricarboxylic acid (TCA) cycle in muscle, liver, heart, kidneys, and brown adipose tissue (Neinast et al. 2019). Interestingly, mice with insulin resistance showed a partial shift of BCAA oxidation in liver and adipose tissue toward muscle (Neinast et al. 2019).

BCAA Catabolism in Adipose Tissue

Altered adipose tissue function in obesity may contribute substantially to the altered circulating metabolite concentrations. A decade ago, Herman et al. provided in vivo evidence that circulating BCAAs are catabolized particularly in adipose tissue, to a greater degree than in skeletal muscle, and that adipose tissue transplantation could lower circulating BCAA levels by 30–50% in mice (Herman et al. 2010). At the

cellular level, in vitro studies have shown that BCAAs are mainly incorporated into proteins during the early stages of adipogenesis, but are oxidized and used for lipid synthesis in later stages of adipocyte development (Estrada-Alcalde et al. 2017). These data add to the evidence from mouse adipocyte cultures showing a switch from glucose and glutamine to BCAA utilization during adipocyte development (Green et al. 2016; Halama et al. 2016).

The mechanisms linking BCAA metabolism and lipid accumulation in adipocytes remain to be fully elucidated. Recent studies have indicated a strong positive relationship between lipid storage and extracellular levels of 3-HIB in adipocyte cultures (Nilsen et al. 2020). Specifically in the valine degradation pathway, 3-HIB is formed by removal of the coenzyme A (CoA) moiety from 3-HIB-CoA, catalyzed by 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) (Shimomura et al. 1994). Free 3-HIB can leave the cell or enter the TCA cycle via further catabolism into methylmalonyl semialdehyde (MMS) toward succinyl-CoA. Consequently, addition of valine increases extracellular 3-HIB concentrations as previously shown in vitro in skeletal muscle cells and hepatocytes (Miyazaki et al. 2015). In adipose cultures, extracellular 3-HIB levels were found to increase during adipocyte differentiation, and knockdown of Hibch lowered extracellular 3-HIB concomitant with lower lipid accumulation, in both white and brown adipocytes (Nilsen et al. 2020). Furthermore, addition of 3-HIB increased fatty acid uptake in both adipocyte subtypes. On the other hand, 3-HIB addition had opposite effects on mitochondrial respiratory capacity and reactive oxygen species (ROS) generation in the white and brown adipocytes, with lower respiration and higher ROS levels in white adipocytes (Nilsen et al. 2020).

3-HIB in Interorgan Metabolic Crosstalk

These data suggest that 3-HIB may function in part as an adipocyte-derived signal, acting locally to promote lipid uptake, and possibly systemically to fine-tune whole-body energy metabolism. In skeletal muscle, 3-HIB was previously found to promote endothelial fatty acid uptake, as a possible mechanism that promotes insulin resistance (Jang et al. 2016). Thus, it is tempting to speculate that adipocyte-derived 3-HIB is involved in metabolic organ crosstalk, in part to facilitate fatty acid uptake and utilization in skeletal muscle and brown adipocytes. In conditions of active fatty acid oxidation in muscle and brown fat, this mechanism could serve to counterbalance increased white adipocyte lipid storage and help maintain energy homeostasis. The marked stepwise increase in circulatory 3-HIB concentrations in prediabetes and type 2 diabetes (Nilsen et al. 2020) might then reflect increased lipid stores, which may drive excessive lipid storage in muscle and brown fat in the absence of concomitant stimulants of fatty acid oxidation.

Although circulating 3-HIB concentrations show strong associations with obesity, insulin resistance, and type 2 diabetes, an important role for 3-HIB as a homeostatic regulator of energy metabolism is supported by several observations. First, circulatory 3-HIB concentrations increase after a 72-h fast (Avogaro and Bier 1989), as well as 6 days after bariatric surgery (Nilsen et al. 2020). A 72-h fast

induces insulin resistance in skeletal muscle, diverting metabolism toward fatty acid oxidation in lieu of glucose oxidation (Vendelbo et al. 2012). Although insulin sensitivity improves 6 days after bariatric surgery (Gudbrandsen et al. 2019) at the same time as 3-HIB increases (Nilsen et al. 2020), followed by a reduction in both 3-HIB and insulin resistance after long-term fat loss at 1 year post-surgery (Nilsen et al. 2020), circulatory triacylglycerol (TAG) concentrations increase or remain unchanged 6 days post-surgery (Gudbrandsen et al. 2019). Thus, increased 3-HIB following 6 days of minimal food intake after surgery likely plays a role in metabolic adaptations. This possibility and the mechanisms involved require further investigation. Second, circulatory 3-HIB concentrations were found to be acutely lowered during a hyperinsulinemic-euglycemic clamp in women, and protein ingestion in the clamp setting, which lowered the insulin-stimulated glucose disposal (i.e., induced insulin resistance), counteracted the decrease in 3-HIB (Harris et al. 2017). Third, 3-HIB was found to incorporate around 15–20% of a bolus of ^{13}C-labeled BCAAs or valine within only 3 min after injection in mice, derived from BCAA metabolism in muscle as well as other tissues (Neinast et al. 2019). Fourth, mice overexpressing the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Pgc-1α) in skeletal muscle, to mimic the molecular effects of exercise, show increased plasma 3-HIB concentrations, particularly in response to a valine bolus (Neinast et al. 2019). Consistently, a human exercise intervention showed a rapid increase in circulating 3-HIB after cycling and a return to baseline after 2 h (Lee et al. 2021). Additionally, 3-HIB was previously also proposed to act as an interorgan metabolite that provides a substrate for hepatic gluconeogenesis via valine degradation in skeletal muscle (Letto et al. 1986). Together, these data demonstrate a rapid and highly dynamic regulation of 3-HIB formation in response to altered nutritional and physiological states.

3-HIB Reflects Hepatic Fatty Acid Oxidation

Metabolic fatty liver, often referred to as nonalcoholic fatty liver disease (NAFLD), is strongly associated with insulin resistance and risk of type 2 diabetes, and this condition can be prevented by increased mitochondrial fatty acid β-oxidation (Barbier-Torres et al. 2020). Potent selective stimulators of hepatic mitochondrial fatty acid β-oxidation may therefore be hypothesized to lower circulatory 3-HIB concentrations. Accordingly, under conditions of pharmacologically induced β-oxidation by synthetic 3-thia fatty acids (TTA), male Wistar rats show a strikingly selective downregulation of hepatic *Hibch* mRNA, among several measured genes involved in BCAA catabolism, and no such effect in skeletal muscle or adipose tissue (Bjune et al. 2021). This selective change in hepatic enzyme expression corresponded to a marked decrease in circulating 3-HIB concentrations. Although the rats were lean and did not show clear changes in body weight or fat mass, these observations point to an important contribution of hepatocytes to circulatory 3-HIB levels, where hepatic 3-HIB efflux increases in conditions of reduced fatty acid oxidation. Of note, no measurements were made of 3-HIB utilization in the TCA cycle or gluconeogenesis,

both of which may use 3-HIB as substrates. However, 3-HIB showed a strong inverse relationship with methylmalonic acid (MMA), downstream of the 3-HIB-derived intermediary metabolite MMS, as circulatory MMA increased strongly upon the activation of fatty acid oxidation (Bjune et al. 2021). In humans, circulatory MMA serves as a marker of vitamin B12 status, and a common missense variant in the *HIBCH* gene shows a strong association with MMA levels (Molloy et al. 2016). Thus, a switch-like mechanism may exist in the HIBCH/3-HIB/MMS pathway, where 3-HIB can either leave the cell or be converted toward MMA and the TCA cycle.

It should be noted that the metabolism of MMA is complex, as it involves B12 activity and is also derived from many other sources including propionyl-CoA derived from the BCAAs leucine and isoleucine. Hence, the cellular mechanisms underlying the inversely associated circulatory levels of 3-HIB and MMA require further investigation. Nonetheless, our experiment with TTA clearly shows that a strong increase in hepatic mitochondrial fatty acid β-oxidation corresponds to lower 3-HIB and higher MMA concentrations in the circulation, associated specifically with *Hibch* expression in the liver and not muscle or adipose tissue. Further studies are needed to elucidate these relationships and mechanisms in humans.

Conclusion and Future Perspectives

Several studies have now documented a marked increase in circulatory 3-HIB concentrations in obesity, insulin resistance, and type 2 diabetes, similar to the BCAAs themselves. However, intermediary metabolites such as 3-HIB might more directly reflect the process of BCAA catabolism, and for 3-HIB specifically in the valine degradation pathway, which may be of clinical value. Although 3-HIB appears to play a key role in the maintenance of normal physiology and energy homeostasis, and may act as a protective signaling substance as well as important metabolic substrate, altered fasting 3-HIB concentrations may provide an early sign of potentially pathological shifts in cellular energy utilization. Circulatory 3-HIB may reflect a shift toward higher white adipocyte lipid storage and decreased muscle/brown fat fatty acid oxidation in obesity and insulin resistance, and generally reduced capacity for mitochondrial fatty acid β-oxidation in the liver as well as other tissues. Further studies are needed to determine the underlying mechanisms and involved tissues that contribute to altered circulatory 3-HIB levels, in different metabolic states and according to specific physiological needs or pathophysiological processes.

Applications to Prognosis, Other Diseases, or Conditions

The available data on 3-HIB so far largely derive from studies in adults, with limited distinction between sexes or race. It will be important to determine to what extent 3-HIB may serve as a useful prognostic marker of insulin resistance and type 2 diabetes for males and females, respectively, as well as for different races and genetic backgrounds. Given the importance of BCAA catabolism in core metabolic

processes, and the role of 3-HIB in lipid and energy homeostasis, assessment of circulatory 3-HIB might also have predictive value for the development of heart disease/cardiovascular diseases and other diseases associated with insulin resistance and aging, including neurodegenerative diseases. 3-HIB can also be detected in saliva, showing positive correlations to 3-HIB levels in serum and increased levels in patients with liver cirrhosis (Miyazaki et al. 2015). Because different cancer cells show distinct changes in BCAA metabolism (Mayers et al. 2016), it is also relevant to consider specific tumors as a source of circulatory and salivary 3-HIB.

Mini-Dictionary of Terms

- **Branched-chain amino acids (BCAAs)**. These are essential amino acids with key roles in core metabolic processes including anaplerotic substrate provision for tricarboxylic acid (TCA) cycle activity. The BCAAs are leucine, isoleucine, and valine.
- **3-Hydroxyisobutyrate (3-HIB)**. This metabolite is an intermediary degradation product of the BCAA valine. Unlike many other BCAA-derived intermediary metabolites, it does not contain a coenzyme A (CoA) moiety and can therefore leave the cell and enter the circulation.
- **Insulin resistance**. Chronic cellular resistance to the peptide hormone insulin manifests in elevated fasting insulin and often eventually also glucose concentrations in the circulation, as more insulin is required to promote insulin-stimulated glucose uptake in target tissues.
- **Fatty acid oxidation**. Fatty acids are sequentially degraded in peroxisomes and mitochondria largely via β-oxidation, which removes 2-carbon acetyl-CoA that are oxidized to CO_2 via the TCA cycle.
- **White adipocyte**. The most abundant type of adipocyte in the body, found in different body fat depots including subcutaneous and visceral adipose tissue, and which has a great capacity for storage of neutral lipids.
- **Brown adipocyte**. A type of adipocyte found in specific areas of the body including the neck, between the shoulder blades, and around the kidneys, with a high number of mitochondria and high capacity for glucose and lipid consumption via adaptive thermogenesis.

Key Facts of Diabetes

Diabetes includes different disease subgroups, including type 1 diabetes, late-onset diabetes of the young (LADA), type 2 diabetes, and monogenic diabetes (e.g., maturity-onset diabetes of the young (MODY)).

More than 450 million people have type 2 diabetes globally.

Type 2 diabetes typically involves overweight/obesity and increased fat deposition in ectopic sites such as the liver and pancreas, which is associated with the development of systemic insulin resistance.

Different pathogenic processes on the cellular level may underlie the development of insulin resistance and type 2 diabetes in different people, according to genetics as well as lifestyle/dietary factors.

Along with insulin resistance, hyperinsulinemia, and eventual loss of sufficient insulin production, a common feature of type 2 diabetes is elevation in circulatory branched-chain amino acids and related intermediary metabolites including 3-HIB.

Type 2 diabetes may be prevented, controlled, and in many cases reversed by consistent dietary energy and carbohydrate restriction, via loss of body fat and prevention of fat (re)gain.

Summary Points

- Obesity and type 2 diabetes are associated with elevated circulatory concentrations of amino acids, in particular the branched-chain amino acids (BCAAs).
- 3-Hydroxyisobutyrate (3-HIB) is an intermediary metabolite in the valine degradation pathway, which is formed via removal of the CoA moiety of 3-HIB-CoA by the enzyme HIBCH.

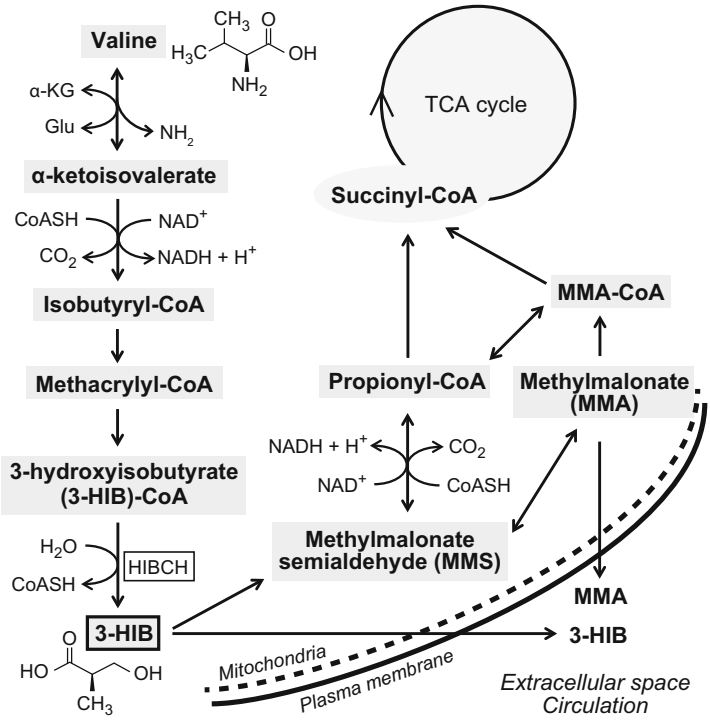

Fig. 1 Schematic overview of the valine-3-HIB pathway. 3-HIB is formed via degradation of the branched-chain amino acid valine. Enzymatic removal of the CoA moiety of 3-HIB-CoA by HIBCH allows 3-HIB to leave the cell and enter the extracellular matrix and circulation

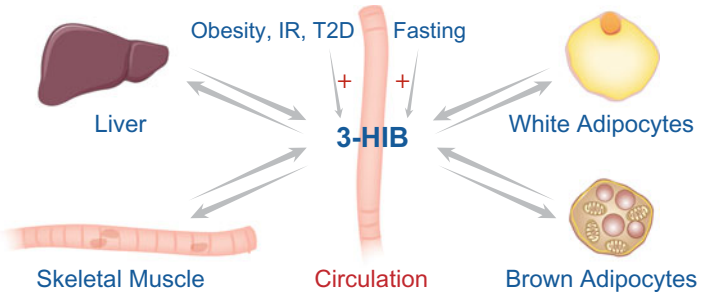

Fig. 2 Putative role of 3-HIB in crosstalk between key metabolic organs. Principal insulin-responsive organs/cells generate 3-HIB, and the resultant extracellular 3-HIB can affect energy and lipid metabolism, locally and likely also distally via the circulation. IR = insulin resistance; T2D = type 2 diabetes

- The lack of CoA allows 3-HIB to leave its cells of origin (e.g., liver, skeletal muscle, adipocytes), giving plasma concentrations around 10–25 μmol/L.
- In prediabetes and type 2 diabetes, defined by elevations in circulatory HbA1c, 3-HIB shows stepwise higher concentrations in plasma, sometimes reaching levels above 30 μmol/L.
- Circulatory 3-HIB concentrations increase also with several days of fasting but decrease with longer-term fat loss.
- Chronic elevations of 3-HIB may reflect a chronic perturbation in energy metabolism that underlies diet-induced pathogenic insulin resistance and type 2 diabetes (Figs. 1 and 2).

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Proteomic Biomarkers: What They Are and How Type 2 Diabetes Mellitus Has Similarities with Other Diseases

14

Karina Braga Gomes

Contents

Introduction 313
Proteomic Biomarkers and Type 2 Diabetes Mellitus 314
Proteomics in Type 2 Diabetes Mellitus Complications 314
Diabetic Retinopathy 315
Diabetic Kidney Disease 315
Diabetic Neuropathy and Chronic Wounds 317
Proteomics in Type 2 Diabetes Mellitus and Other Diseases 317
Type 2 Diabetes Mellitus and Obesity 317
Type 2 Diabetes Mellitus and Cardiovascular Diseases 320
Type 2 Diabetes Mellitus and Metabolic Diseases 321
Type 2 Diabetes Mellitus and Pancreatic Cancer 322
Type 2 Diabetes Mellitus and Neurologic Disorders 322
Conclusion 324
Applications to Prognosis and Other Diseases or Conditions 324
Applications to Prognosis 324
Applications to Other Diseases or Conditions 324
Mini-Dictionary of Terms 324
Key Factors of Proteomic Biomarkers in Diabetes Mellitus 325
Summary Points 325
References 326

Abstract

Type 2 diabetes mellitus (T2DM) is the most frequent form of diabetes, characterized by insulin resistance and impaired insulin release. Its prevalence has shown remarkable expansion worldwide, particularly in low- and middle-income countries. Diabetic complications comprise kidney disease, retinopathy, and

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neuropathy. Moreover, metabolic, cardiovascular, and neurologic diseases have shared common pathways with T2DM. Proteomics is a technology involved in the quantification of overall proteins present in biological samples, as well as the study of their structures, functions, and interactions. Consequently, proteomics could be considered as the most relevant information to characterize a biological system and to propose new candidate biomarkers. This chapter goes on to discuss proteomic studies in order to characterize the T2DM profile, as well as the proteins commonly observed in T2DM, its complications, and other diseases.

Keywords

Type 2 diabetes mellitus · Proteomics · Biomarkers · Complications · Mass spectrometry · Retinopathy · Diabetic kidney disease · Neuropathy · Chronic wounds · Metabolic diseases · Obesity · Cancer · Alzheimer's disease

Abbreviations

2D PAGE	Two-dimensional polyacrylamide gel electrophoresis
AD	Alzheimer's disease
AGEs	Advanced glycation end products
Apo	Apolipoprotein
CD163	Scavenger receptor cysteine-rich type 1 protein M130
CFAH	Complement factor H
CILP2	Cartilage intermediate layer protein 2
CKD	Chronic kidney disease
CTSD	Cathepsin D
CVDs	Cardiovascular diseases
DAF	Decay-accelerating factor
DKD	Diabetic kidney disease
DM	Diabetes mellitus
eGFR	Estimated glomerular filtration rate
eGPx	Extracellular glutathione peroxidase
ELISA	Enzyme-linked immunosorbent assays
GAL4	Galectin-4
Hb1Ac	Glycated hemoglobin A1c
HFpEF	Heart failure with preserved ejection fraction
HOMA-IR	Homeostatic model assessment of insulin resistance
IGFB2	Insulin-like growth factor-binding protein 2
InR	Insulin-like receptor
L1CAM	L1 cell adhesion molecule
LC	Liquid chromatography
MCI	Mild cognitive impairment
MetS	Metabolic syndrome
MMPs	Matrix metalloproteinases
MS	Mass spectrometry
mRNA	Messenger ribonucleic acid

NAFLD	Non-alcoholic fatty liver disease
NGAL	Neutrophil gelatinase-associated lipocalin
OR	Odds ratio
PAD	Peripheral artery disease
PIP	Prolactin-induced protein
PMCA	Plasma membrane Ca + 2 ATPase
RLS	Restless leg syndrome
STAT	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
THBS2	Thrombospondin-2

Introduction

In the last decades, the prevalence of diabetes mellitus (DM) has shown a remarkable increase worldwide, particularly the type 2 diabetes mellitus (T2DM) (Cheema et al. 2020). The number of people with DM has increased more rapidly in low- and middle-income countries compared to high-income countries (WHO 2021). T2DM is the most frequent form of diabetes, characterized by insulin resistance and impaired insulin release by pancreatic β-cells (Cheema et al. 2020). Complications of DM comprise diabetic kidney disease (DKD, the major cause of renal failure), retinopathy, and neuropathy. Moreover, cardiovascular diseases (CVDs) are the main comorbidity observed in diabetic patients (Zarch et al. 2020).

Biomarker is a biological compound whose state or level determines the normal or pathological status associated with a disease or treatment response. The proteins, mainly the soluble ones, are important biomarkers because they allow the laboratory measurement to prognosis and diagnosis of acute and chronic disorders (Sohail et al. 2018). The molecular omics platforms, as proteomics, permit the identification of multi-parameter biomarkers and the development of personalized diagnostics. Nevertheless, applications of these omics technologies should attempt to clinical validation of the candidate biomarkers in huge populations, one of the main challenges in omics research (Tans et al. 2019).

Proteomics is a technology involved in the quantification of overall proteins present in a cell, tissue, or organism. The proteome characterization includes protein expression, as well as its structure and function, besides interactions and post-translational modifications. Proteins are regulators of biological function, and their levels are dependent on gene expression and mRNA (messenger ribonucleic acid) levels, as well as translational control (Aslam et al. 2017). Proteomes are more complex than the corresponding genomes, because post-translational modifications greatly contribute to a larger diversity than genes (Zhang et al. 2014). Thus, the proteomics could be considered as the most relevant information to characterize a biological system and to propose new biomarkers (Aslam et al. 2017).

Immuno-based assays that utilize the affinity of antibodies linked to a reporter (such as enzyme-linked immunosorbent assays (ELISA), luminescence, fluorescence, or radioactivity) are considered the gold standard for identification and quantification of

proteins. These assays were utilized to prove the association of classic T2DM biomarkers, such as adiponectin, leptin, sex hormone-binding globulin, and vitamin E-binding protein. Nevertheless, these assays are specific for only one biomarker and do not allow untargeted measure of proteins present in the sample (Chen and Gerszten 2020). Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been used as a reference methodology for proteomic studies. However, gel-based techniques tend to be more labor-intensive and time-consuming, consequently not suitable for high-throughput analysis (Zhang et al. 2014). In turn, mass spectrometry (MS) has been considered the most important method to identify, characterize, and quantify proteins on a large scale. Because of the large variety of relative abundances and complex components commonly present in biological samples, MS allows the detection and quantification of low-abundance species based on mass-to-charge ratio (m/z), since most serum proteins are at or below the limit of detection for most immune assays. Liquid chromatography (LC) also allows continuous separation of thousands of proteins and can be performed online with MS for increased throughput (Zhang et al. 2014).

Proteomic Biomarkers and Type 2 Diabetes Mellitus

Several studies have shown that proteomic analysis contributes to the identification of serum, urinary, salivary, and other tissue-specific protein biomarkers in DM (Riaz et al. 2010a; Kraniotou et al. 2018; Tans et al. 2019), which provides insights related to disease prognosis, likelihood of diabetes progression, development of complications, as well as therapy response (Chen and Gerszten 2020). Proteins related to abdominal adiposity, insulin resistance, and homeostatic model assessment of insulin resistance (HOMA-IR) have been shown to be associated with T2DM in many studies. Moreover, serpin peptidase inhibitor A1, growth-inhibiting protein 25, apolipoprotein A-1, galectin-1, haptoglobin, transthyretin, zinc-alpha 2-glycoprotein, prealbumin, thiol-specific antioxidant, tissue plasminogen activator, retinol-binding protein 4, cathepsin D, interleukin-1 receptor antagonist, fatty acid-binding protein 4, complement C3f, and fragments of kininogen 1 precursor were also associated with T2DM (Nowak et al. 2016; Meng et al. 2017; Sohail et al. 2018; Kim et al. 2019; Chen and Gerszten 2020). Furthermore, insulin-like growth factor-binding protein 2 levels were lower in T2DM patients compared to controls, which was also associated with a higher Hb1Ac (glycated hemoglobin A1c) levels (Noordam et al. 2020).

Proteomics in Type 2 Diabetes Mellitus Complications

Urine, tears, and blood proteome profile have been used to determine biomarkers for T2DM associated with complications, whose origin is the advanced glycation end products (AGEs) formation. Some studies showed that hyperglycemia is positively correlated with TNF-alpha and Beta-2 microglobulin serum levels, which suggests that the progression of T2DM and its complications, mainly DKD and retinopathy, are directly associated with inflammatory and vascular proteins (Sohail et al. 2018).

Diabetic Retinopathy

Diabetic retinopathy is one of the most common microvascular complications of T2DM. Vascular endothelial growth factor, angiotensin-converting enzyme, insulin-like growth factor, AGEs, and antiangiogenic factors are aspects related to physiopathology of retinopathy. Recently, a study presented the association of salivary biomarkers with diabetic retinopathy and its severity by MS. The autohrs observed 119 differentially expressed proteins in proliferative diabetic retinopathy group compared to non-proliferative diabetic retinopathy group, which were involved on pathways indicating increased farnesoid X receptor/retinoid X receptor activation, liver X receptor/retinoid X receptor activation, sucrose degradation V, acute phase response signaling, and regulation of actin-based motility by Rho (Chee et al. 2016).

This study showed the saliva as a possible alternative sample source for proteomic analysis related to retinopathy (Chee et al. 2016). In addition, Midena et al. (2021) applied a liquid biopsy using aqueous and vitreous humor from diabetic patients and observed, by proteomics and metabolomics analysis, that neurodegeneration, neuroinflammation, and vasculopathy are detectable in these intraocular fluids. Moreover, the concentrations of proteins and metabolites change in different stages of disease and in response to treatment, mainly in diabetic macular edema and proliferative retinopathy (Midena et al. 2021).

Another study showed that 111 proteins were downregulated, and 80 were upregulated in the aqueous humor of proliferative diabetic retinopathy patients compared to controls (senile cataract). These proteins were related to several pathways, as complement and coagulation cascades, platelet activation, focal adhesion, protein digestion and absorption, extracellular matrix-receptor interaction, human papillomavirus infection, PI3K-Akt signaling pathway, peroxisome proliferator-activated receptor signaling pathways, cholesterol metabolism, vitamin digestion/absorption, and fat digestion/absorption pathways (Xiao et al. 2021). A panel comprising four plasma proteins – apolipoprotein (Apo) AIV, complement C7, clusterin, and inter-alpha-trypsin inhibitor heavy chain H2 – was also related to early stages of diabetic retinopathy (Jin et al. 2016). Moreover, beta 2-glycoprotein I, alpha 2-HS-glycoprotein, alpha 1-acid glycoprotein, and apolipoprotein A-1 presented lower abundance in diabetic retinopathy compared to control group, but beta 2-glycoprotein I expression was gradually increased during the development of diabetic retinopathy (Liu et al. 2011).

Diabetic Kidney Disease

The physiopathology of DKD or nephropathy is complex, because prolonged hyperglycemia can provide activation of different proteins and signaling pathways, with severe morphological changes in the kidney. It is known that low concentration of urinary albumin (microalbuminuria) is detectable only when the functional impairment in the glomerulus is already in progress, but it is not enough to prevent the onset of DKD. Consequently, a proteomic analysis is important to afford new

biomarkers using blood or urine without other invasive techniques (Conserva et al. 2013). As urinary proteins are derived from a variety of sources, such as plasma, secreted by the kidney, metabolism, derived from podocytes, tubules, and endosome, the urine sample became an important font to proteomic studies (Merchant and Klein 2005).

Papale et al. (2010) showed that ubiquitin and β2-microglobulin were differentially expressed in the urine of patients with DKD, whose results were reinforced by renal biopsies. Another study showed a reduction in collagen fragments previously the onset of macroalbuminuria, which suggest that collagen could be a marker to predict progression of DKD (Zürbig et al. 2012), which was corroborated by Maahs et al. (2010) (24). Moreover, Jim et al. (2012) presented that small traces of nephrin were found in 50% of the T2DM patients with normoalbuminuria, suggesting the role of nephrin as an early biomarker of DKD. Furthermore, extracellular glutathione peroxidase (eGPx) and ApoE were found to exhibit a progressive reduction in T2DM patients with microalbuminuria and chronic renal failure (Kim et al. 2007).

Combining two-dimensional liquid chromatography/MS analysis and enzyme-linked immunosorbent assay in urinary samples, Riaz et al. (2010b) showed that transthyretin, haptoglobin precursor, and α-1-microglobulin/bikunin precursor levels decreased 30.8%, 81.45%, and 55.2%, while albumin, zinc α2 glycoprotein, E-cadherin, and retinol-binding protein 4 levels increased 486.5%, 29.23%, 693%, and 100%, respectively, in T2DM patients compared to controls. Golea-Secara et al. (2020) also investigated urinary peptides and observed that apolipoprotein AI, neutrophil gelatinase-associated lipocalin, cytidine deaminase, S100 calcium-binding protein A8, and hemoglobin subunit delta were associated with early DKD. Upregulated fragments of blood components in urine samples of patients with DKD, such as alpha-1-antitrypsin, albumin, transthyretin, alpha-2-HS-glycoprotein, and beta-2-microglobulin, were also observed according to Alkhalaf et al. (2010). Only the collagen fragment was downregulated in urine samples of these patients (Alkhalaf et al. 2010). Differentially expressed proteins were also identified in urine from normoalbuminuric and microalbuminuric T2DM patients, such as alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen (Jin et al. 2012).

Another proteomic analysis identified higher plasma levels of prolactin-induced protein (PIP), thrombospondin-2 (THBS2), L1 cell adhesion molecule (L1CAM), and neutrophil gelatinase-associated lipocalin (NGAL) in T2DM patients, whereas PIP, THBS2, and NGAL were significantly higher in T2DM patients with albuminuria, while L1CAM levels were higher in T2DM patients with retinopathy (Yeh et al. 2016). Upregulated glycoproteins, such as lumican, vasorin, and retinol-binding protein 4, were also observed in T2DM with DKD using plasma samples (Bellei et al. 2008). The decreased levels of prostatic acid phosphatase precursor, ribonuclease, and kallikrein-3, as well as progressive increase of transthyretin precursor, Ig κ-chain C region, Ig κ-chain V-II region Cum, Ig κ-chain V-III region SIE, carbonic anhydrase 1, plasma retinol-binding protein, β-2-microglobulin precursor, and β-2-glycoprotein 1 levels, were observed in normoalbuminuric T2DM and DKD patients (Ahn et al. 2010).

An untargeted proteomic study, with functional analysis, showed that differentially expressed urinary proteins in patients with biopsy-proven DKD were associated with complement and coagulation pathways. Urinary protein quantification showed that C3, C9, and complement factor H (CFAH) correlated negatively with estimated glomerular filtration rate (eGFR) decline evaluated annually; and urinary abundances of C5, decay-accelerating factor (DAF), and CD59 correlated positively with rate of eGFR decline in this same period. In addition, higher CFAH and lower DAF urinary were independently associated with higher risk of progression to end-stage renal disease (Zhao et al. 2021).

Diabetic Neuropathy and Chronic Wounds

Few studies have evaluated the proteomics of diabetic peripheral neuropathy (DPN), a common and severe complication of T2DM. Ising et al. (2021), using peripheral nerves, evaluated the proteins involved in decreased sural nerve action potential in T2DM patients compared to controls. A total of 2617 proteins were identified. A cluster of 500 proteins was able to differentiate T2DM subjects and healthy controls, which was related to DPN development (Ising et al. 2021).

Healing of injured tissue is a complex and impaired process in DM. A study comparing protein composition of chronic diabetic foot exudates to exudates from sites of burn healthy victims observed increased expression of S100 calcium-binding protein A8/A9. Moreover, matrix metalloproteinases (MMPs) MMP1, MMP2, and MMP8 were observed to be elevated in chronic wounds from diabetics with significant impact on collagen degradation and tissue destruction. Finally, Annexin A5 was exclusively found in chronic wound exudates from DM patients (Krisp et al. 2013).

Proteomics in Type 2 Diabetes Mellitus and Other Diseases

Several studies have investigated proteins commonly expressed in T2DM and other diseases (Table 1).

Type 2 Diabetes Mellitus and Obesity

The molecular mechanism underlying obesity and T2DM is not completely known. However, proteomics has helped to advance our understanding about the origin, onset, development, prevention, and treatment of these complex diseases (Alfadda et al. 2013). Alfadda et al. (2013) observed that aldo-keto reductase 1 C2, protein disulfide isomerase, prohibitin 1, profilin, beta actin, alpha crystallin B, and annexins A1, A5, and A6 were highly expressed in adipocytes from old compared to young obese patients. However, keratin type 2 cytoskeletal 1, keratin type 2 cytoskeletal 10, and hemoglobins A and B were less abundant in the first group. Moreover, signal

Table 1 Proteomic biomarkers differently expressed in type 2 diabetes mellitus and other diseases

Obesity	Cardiovascular diseases	Metabolic syndrome	Gout	Pancreatic cancer	Restless leg syndrome	Alzheimer's disease/mild cognitive impairment
Aldo-keto reductase 1 C2 disulfide isomerase Prohibitin 1 Profilin Beta actin Alpha crystallin B Annexin A1 Annexin A5 Annexin A6 Keratin type 2 Cytoskeletal 1 Keratin type 2 cytoskeletal Hemoglobin A Hemoglobin B Signal transducer and activator of transcription 3 Prohibitin 1 Keratin type 2 cytoskeletal 10 Insulin effectors secreted decoy of insulin-like receptor (InR) Protein kinase C-binding protein LDL receptor-related protein 6	Retinol-binding protein Glutathione peroxidase-3 Fatty acid-binding protein 4 Scavenger receptor cysteine-rich type 1 protein M130 Insulin-like growth factor-binding protein 2 Cathepsin D Galectin-4 Midkine Kidney injury molecule-1 Interleukin-23 Follicle-stimulating hormone Angiopoietin-1 Eotaxin-1 γ1-laminin β2-laminin α1-type IV collagen α2-type IV collagen fatty acid-binding protein Alpha-1-	Insulin-like growth factor-binding protein 2 Leptin	CXCL8	S100 calcium-binding protein A9 Aldehyde dehydrogenase 2 family	Apolipoprotein C-II Alpha-1 antitrypsin Haptoglobin Fibrinogen alpha chain Platelet factor 4 Plasma protease C1 inhibitor proteins	Haptoglobin Transthyretin Ig α-1 chain C region Ig μ chain C region Ig κ chain C region Apolipoprotein A-I Apolipoprotein A-IV Apolipoprotein B-100 Apolipoprotein E Galectin-3-binding protein Ceruloplasmin Alpha-2 macroglobulin Complement C4 Inter-alpha-trypsin inhibitor heavy chain 1 Inter-alpha-trypsin inhibitor heavy chain 2 Pancreatic polypeptide Zinc alpha 2-glycoprotein Butyrylcholinesterase Acetylcholinesterase Glycogen synthase kinase-3 Glucagon-like peptide-1 Caspase 3 Insulin-like growth factor 1 Catalase Tumor necrosis factor Leptin Vascular endothelial growth factor A Interleukin-6 Interleukin-10 Factor VII

Plasma membrane Ca + 2 ATPase)-interacting single-PDZ protein PDZ domain-containing protein 11 Cordon-bleu Moesin Zinc finger protein 611 78 kDa glucose-regulated protein Cytochrome c oxidase subunit 6B1	microglobulin/ bikunin precursor Trafficking protein particle complex subunit 3 Pigment epithelium-derived factor Tumor necrosis factor ligand superfamily member 15 Ubiquitin-conjugating enzyme E2 G2 Reticulon-4 receptor Insulin Cartilage intermediate layer protein 2 Apolipoprotein M					C-reactive protein Fatty acid-binding protein Amyloid A1 Pappalysin Glucose regulatory protein 78 Valosin-containing protein Calreticulin Ubiquinol-cytochrome c reductase core protein Disulfide isomerase DnaK Collapsin response mediator protein 2 Glutamine synthetase Proprotein convertase subtilisin Chaperonin 60 T-complex protein 1 Peroxiredoxin L-3-hydroxyacyl-coenzyme A dehydrogenase Secretagogin

transducer and activator of transcription (STAT) 3 was identified as the central molecule involved in this disease. These proteins are related to apoptosis, cellular senescence, and inflammatory responses, which are also commonly observed in DM (Alfadda et al. 2013). Interestingly, proteins prohibitin 1 and keratin type 2 cytoskeletal 10 are described as biomarkers for T2DM at diagnosis in obese patients (López-Villar et al. 2015).

Comparing liver samples from diabetic and non-diabetic morbid obese subjects, Valle et al. (2012) analyzed 850 proteins. They observed that 27 proteins were differentially expressed in T2DM obese subjects, whose pattern was decreased abundance in mitochondrial enzymes, proteins involved in methionine metabolism, and oxidative stress response. T2DM subjects also presented decrease of glutathione and higher levels of protein and lipid oxidative damage. Changes in proteasome subunits, retinoic acid synthesis, detoxifying enzymes, and carbohydrate metabolism were also found in this group (Valle et al. 2012).

Insulin-induced intracellular signaling pathway is very important in metabolic diseases and cellular processes related to DM. The insulin receptor and its substrates are necessary in the insulin signaling network, which insulin binds to its receptor to generate tyrosine phosphorylation cascades that consequently activate other cascades (López-Villar et al. 2015). A phosphoproteomic analysis in brown adipocytes found that the insulin effectors secreted decoy of insulin-like receptor (InR), protein kinase C binding protein, LDL receptor-related protein 6, and PMCA (plasma membrane Ca + 2 ATPase)-interacting single-PDZ protein/PDZ domain-containing protein 11, a potential calcium ATPase-binding protein, which are also involved in insulin signaling related to obesity (Krϵuger et al. 2008; López-Villar et al. 2015). Furthermore, protein cordon-bleu, moesin, zinc finger protein 611, 78 kDa glucose-regulated protein, and cytochrome c oxidase subunit 6B1 were differentially expressed in diabetic group compared to non-diabetic group in subcutaneous and visceral adipose tissue obtained from patients with morbid obesity (Fang et al. 2015).

Type 2 Diabetes Mellitus and Cardiovascular Diseases

Patients with T2DM are at considerable risk of developing cardiovascular disease due to both microvascular and macrovascular complications. T2DM patients with cardiovascular disease showed higher expression of plasma retinol-binding protein and glutathione peroxidase-3 compared to those without cardiovascular disease and non-diabetic controls, suggesting that inflammatory and redox state influence the pathogenesis of the cardiovascular disease in T2DM patients (García-Fontana et al. 2015). A study investigated the association of proteins previously shown to be related to diabetes [fatty acid-binding protein 4 (FABP4), scavenger receptor cysteine-rich type 1 protein M130 (CD163), insulin-like growth factor-binding protein 2 (IGFB2), cathepsin D (CTSD), plasminogen activator inhibitor 1, paraoxonase-3, and galectin-4 (GAL4)] with all-cause mortality, cardiovascular mortality, incident coronary events, and incident heart failure. Significant associations were identified between CD163, IGFBP2, GAL4, CTSD, and FABP4 with all-cause

mortality; CTSD, CD163, GAL4, and IGFBP2 with cardiovascular mortality; GAL4, FABP4, and CTSD with coronary events; and GAL4 and CTSD with heart failure (Molvin et al. 2020).

Peripheral artery disease (PAD) is one of the most prevalent macrovascular complications of DM, which affects 20–30% of patients, and is associated with a higher risk of fatal and non-fatal cardiovascular/cerebrovascular events (Fang et al. 2015). McCarthy et al. (2019) proposed a proteomics model to predict PAD in diabetic patients composed by midkine, kidney injury molecule-1, interleukin-23, follicle-stimulating hormone, angiopoietin-1, and eotaxin-1. Associated with history of hypertension, the model showed sensitivity of 84%, specificity of 75%, positive predictive value of 84%, and negative predictive value of 75% for PAD detection among patients with DM. Besides, abnormal scores in this model were associated with a shorter time to revascularization during 4.3 years follow-up (McCarthy et al. 2019).

Preil et al. (2015) analyzed non-atherosclerotic arteries collected at coronary bypass operations from patients with T2DM and controls. Basement membrane components, such as γ1-laminin, β2-laminin, α1-type IV collagen, and α2-type IV collagen, were significantly increased in patients with diabetes mellitus. In addition, the expressions of these basement membrane components were significantly lower in metformin users compared to non-users.

DM is also associated with a higher risk of hospitalization and mortality in patients with heart failure with preserved ejection fraction (HFpEF). Firstly, using univariate models of proteomic analysis, Hanff et al. (2021) observed that fatty acid-binding protein, alpha-1-microglobulin/bikunin precursor, trafficking protein particle complex subunit 3, pigment epithelium-derived factor, tumor necrosis factor ligand superfamily member 15, ubiquitin-conjugating enzyme E2 G2, reticulon-4 receptor, insulin, cartilage intermediate layer protein 2 (CILP2), and apolipoprotein M (ApoM) had significantly different expressions between participants with or without diabetes and HFpEF. Proteins maintained after covariate adjustment were alpha-1-microglobulin/bikunin precursor protein, which was higher in DM, and ApoM and CILP2, which are lower in DM. Finally, ApoM was significantly associated with heart failure hospitalization, the time to cardiovascular death, and aborted cardiac arrest (Hanff et al. 2021).

Type 2 Diabetes Mellitus and Metabolic Diseases

The metabolic syndrome (MetS) is a complex disease associated with T2DM and cardiovascular diseases, especially coronary artery disease, stroke, and heart failure. Its characteristics are increased waist circumference, hypertriglyceridemia, reduced high-density lipoprotein, hyperglycemia, and increased blood pressure (Elhadad et al. 2021). A study showed association (lowest odds ratio, OR) of insulin-like growth factor-binding protein 2 (IGFBP2) with MetS and leptin with the highest OR. IGFBP2 was the protein most strongly associated, and plasminogen activator inhibitor 1 had the largest magnitude of association with incident MeS. The best

proteins as predictive MetS biomarkers were netrin receptor and aminoacylase-1 (Elhadad et al. 2021).

Performing a proteome-wide analysis in patients with gout, a disease associated with metabolic syndrome and cardiovascular disease, Kienhorst et al. (2015) found high levels of the chemokine CXCL8 in these patients associated with diabetes mellitus. Besides, the role of immunologic pathways in T2DM was also observed by Abdulwahab et al. (2019), who found 62 proteins differentially expressed in T2DM compared with control subjects, mainly associated with upregulation of immunoglobulins.

Type 2 Diabetes Mellitus and Pancreatic Cancer

Diabetes mellitus is diagnosed in more than 80% of the pancreatic tumor cases (Fogar et al. 1994). Basso et al. (2005) evaluated proteins from pancreatic cancer cell lines and compared them with proteomics of blood from controls and chronic pancreatitis patients. They observed the presence of the S-100 calcium-binding protein only in samples from diabetic patients, which was suggested to be a pancreatic cancer-DM associated. In order to investigate the pancreatic cancer associated to DM, Wang et al. (2013) found 12 upregulated and 11 downregulated proteins in pancreatic cancer tissues from DM patients compared to controls. The proteins S100 calcium-binding protein A9 and aldehyde dehydrogenase 2 family were selected to be evaluated in other population, and the results were confirmed and validated.

Type 2 Diabetes Mellitus and Neurologic Disorders

Restless leg syndrome (RLS) is a neurologic disorder, considered one of the most common sleep-related movement diseases. Mondello et al. (2021), comparing RLS patients and controls, observed a pathway differentially expressed in the first group, compounded by apolipoprotein C-II, alpha-1 antitrypsin, haptoglobin, fibrinogen alpha chain, platelet factor 4, and plasma protease C1 inhibitor proteins, which were associated with the DM and its complications in other studies, suggesting a linked between RLS and DM.

Alzheimer's disease (AD) is the most common type of dementia, characterized by deposition of β-amyloid peptide, hyperphosphorylation of tau protein, neuronal loss, and neuroinflammation. Several studies suggest a relationship between AD and T2DM, although the common pathophysiological mechanisms of this interrelation are still unclear. There are some hypotheses to explain the association between AD and T2DM: hyperglycemia, which leads to glutamate-induced excitotoxicity in neuronal cells; insulin resistance, which may contribute to amyloid-β accumulation in the brain, tau phosphorylation, AGE formation, oxidative stress, and apoptosis; and inflammation, as a common pathway between these diseases (Santos et al. 2017). Moreover, hyperinsulinemia and hyperamylinemia lead to proteotoxicity and β-amyloid protein deposition (Avila-Vazquez et al. 2017).

Recently, we conducted a systematic review of studies that used proteomic methodologies for the identification of common plasma/serum proteins in AD and T2DM (Pereira et al. 2021). Our search on different databases returned 432 articles about proteomics in AD and 317 in T2DM. After applying the inclusion and exclusion criteria, as well as the quality score, 22 studies about AD and 12 about T2DM were included, resulting in 1,185 AD cases versus 1,678 controls and 834 T2DM cases versus 2,500 controls. The studies identified 205 proteomic biomarkers for AD and 149 for T2DM. An overlap was carried out in these studies, and 17 proteomic biomarkers were differentially expressed in both diseases: haptoglobin, transthyretin, Ig α-1 chain C region, Ig μ chain C region, Ig κ chain C region, apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein B-100, apolipoprotein E, galectin-3-binding protein, ceruloplasmin, alpha-2 macroglobulin, complement C4, inter-alpha-trypsin inhibitor heavy chain 1, inter-alpha-trypsin inhibitor heavy chain 2, pancreatic polypeptide, and zinc alpha 2-glycoprotein. These proteins are involved in cholesterol metabolism, vitamin digestion and absorption, fat digestion and absorption, complement and coagulation cascade pathways (Pereira et al. 2021).

Another narrative review highlighted four proteins commonly involved in T2DM and AD: butyrylcholinesterase, acetylcholinesterase, glycogen synthase kinase-3, and glucagon-like peptide-1. Besides, amylin, an amyloidogenic/apoptotic cell death in insulin-producing β-cell inducer, was found in both AD and T2DM (Mirza et al. 2014).

Common differential expression of caspase 3, insulin-like growth factor 1, catalase, tumor necrosis factor, leptin, vascular endothelial growth factor A, and interleukin-6 in both AD and T2DM were also observed, as well as the correlation of these biomarkers with non-alcoholic fatty liver disease (NAFLD) (Gholizadeh et al. 2020).

It is known that DM is one of the most important risk factors for chronic kidney disease (CKD). Examining the relationship between eGFR and neuropsychological tests, Szerlip et al. (2015) observed that lower eGFR levels were associated with worst neuropsychological scores. In addition, serum interleukin-10, factor VII, C-reactive protein, and fatty acid-binding protein proteomic profile were related to CKD-related mild cognitive impairment (MCI), a prodromal AD stage (Szerlip et al. 2015).

Interestingly, a case control study compared the AD-related proteomic profile between T2DM and controls ruled out as having milder prolonged hypoglycemia versus severe transient hypoglycemia. The results showed that, although no differential protein changes were observed in T2DM group, milder prolonged hypoglycemia caused greater AD-related protein changes than severe acute hypoglycemia in control subjects, based on amyloid A1 and pappalysin level changes (Moin et al. 2021).

A proteomic analysis of islets was carried out to determine the protein constituents of normal adult mouse islets (Nicolls et al. 2003). An important subset of the proteins identified in pancreatic islets was associated with AD pathogenesis, such as glucose regulatory protein 78, valosin-containing protein, calreticulin, ubiquinol-cytochrome c reductase core protein, protein disulfide isomerase, DnaK, collapsin response mediator protein 2, glutamine synthetase, proprotein convertase subtilisin, chaperonin 60, T-complex protein 1, peroxiredoxin, L-3-hydroxyacyl-coenzyme A dehydrogenase, and secretagogin, suggesting that these proteins play roles in both AD and type 2 diabetes (Nicolls et al. 2003).

Conclusion

Personalized medicine has the potential to improve T2DM management. The applicability of proteomics in the development of novel biomarkers in this context will contribute to T2DM diagnostics and the comprehension of its relationship with other diseases. Moreover, proteomics use will contribute to improvement in healthcare quality, identification of early-stage patients, and the implementation of preventive measures of T2DM complications. Consequently, proteomic profiles should be integrated to current healthcare systems in order to reduce the complications and delay the T2DM onset.

Applications to Prognosis and Other Diseases or Conditions

Applications to Prognosis

In this chapter, we discussed that urine, tears, and blood proteome profiles have been used to determine biomarkers for T2DM complications, whose origin is the advanced glycation end product formation. Diabetic retinopathy is one of the most common microvascular complications of T2DM, and studies showed protein profiles associated with neurodegeneration, neuroinflammation, and vasculopathy in retinopathy, which are detectable in intraocular fluids (Liu et al. 2011). In addition, it is known that microalbuminuria is detectable only when the functional impairment in the glomerulus is already in progress. Consequently, a proteomic analysis is important to afford new biomarkers using blood or urine without other invasive techniques to detect diabetic kidney disease early (Golea-Secara et al. 2020). The proteomics of diabetic peripheral neuropathy, a common and severe complication of T2DM, is an important tool to prognosis and treatment of this complication (Ising et al. 2021).

Applications to Other Diseases or Conditions

We reviewed studies that showed T2DM proteomic profile in association with other diseases, such as obesity (López-Villar et al. 2015), cardiovascular diseases (Molvin et al. 2020), metabolic syndrome (Elhadad et al. 2021), pancreatic cancer (Basso et al. 2005), and Alzheimer's disease (Pereira et al. 2021), which suggest common pathways between T2DM and these disorders.

Mini-Dictionary of Terms

Type 2 diabetes mellitus – the most frequent form of diabetes, characterized by insulin resistance and impaired insulin release by pancreatic β-cells

Proteomics – technology involved in the quantification of overall proteins present in a cell, tissue, or organism

Complications of diabetes – long-term problems that can develop gradually, related to advanced glycation end products, and can lead to serious damage if not treated

Metabolic syndrome – disease characterized by increased waist circumference, hypertriglyceridemia, reduced high-density lipoprotein, hyperglycemia, and increased blood pressure

Restless leg syndrome – a neurologic disorder, considered one of the most common sleep-related movement diseases, where the main symptom is an overwhelming urge to move your legs

Alzheimer's disease: the most frequent type of dementia, characterized by memory impairment, followed by progressive decay in other cognitive skills, leading to functional decline and loss of autonomy

Key Factors of Proteomic Biomarkers in Diabetes Mellitus

Biomarkers are biological compounds whose state or level determines the normal or pathological status associated with a disease, prognosis, or treatment response.

Proteins, mainly the soluble ones, are one of the most important biomarkers because they allow the laboratory measurement to prognosis and diagnosis of acute and chronic disorders.

Proteomics is a technology that evaluates protein expression and its structure and function, interactions, and post-translational modifications. Proteomes are more complex than the corresponding genomes, because post-translational modifications greatly contribute to the much larger diversity than genes.

Diabetes mellitus, a chronic metabolic noncommunicable disease, has reached epidemic proportions worldwide. More than 95% of all individuals with diabetes mellitus have type 2 diabetes mellitus (T2DM).

Proteomic technology should be integrated to current healthcare systems in order to reduce the complications and delay the T2DM onset, as well as to improve T2DM management.

Summary Points

Proteomics is a technology that allows the quantification of biomarkers (proteins) in type 2 diabetes mellitus (T2DM).

Some protein profiles are associated with T2DM development.

Proteomics can be used to T2DM prognosis, mainly related to diabetic complications: kidney disease, retinopathy, and neuropathy.

Protein profiles associated with T2DM can be observed in other diabetes-related diseases, such as obesity, metabolic syndrome, and cardiovascular diseases.

Neurologic disorders and cancer can share proteomic features with T2DM.

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Salivary C-Reactive Protein as a Biomarker and Implications for Diabetes

15

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Contents

Introduction 331
Biomarkers of Type 2 Diabetes 331
C-Reactive Protein and Pathogenesis 333
Type 2 Diabetes and CRP 334
Human Saliva as Diagnostic Fluid for Biomarkers 334
Salivary Biomarkers in Type 2 Diabetes 335
Identification of Diabetes-Associated Serum Proteins Found in Saliva 335
Measurement of Diabetes-Related Serum Protein Markers in Saliva of Diabetic Patients 337
Correlation of the CRP Levels in Saliva and Serum 337
Diagnostic Methods of Salivary Biomarker Identification 339
Enzyme-Linked Immunosorbent Assay (ELISA) 339
Multiplex Analysis 340
Other Detection Methods 341
Applications to Prognosis, Other Diseases, or Conditions 341
Mini-dictionary of Terms 342
Key Facts of C-Reactive Protein 342
Summary Points 343
References 343

Abstract

C-reactive protein (CRP) is an acute-phase protein synthesized by the liver due to inflammation. Higher CRP concentration has been linked to the increased risk of developing diabetes. Saliva is a biofluid rich in more analytes, proteins, peptides, and an alternative for serum/plasma to identify disease markers. Increased salivary CRP concentration is due to the low-grade inflammation associated with

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type 2-diabetes (T2D). This chapter focuses on the extent of CRP in different pathogenic conditions, explicitly discussing the CRP relationship with diabetes. The circulating CRP is used as a biomarker to diagnose the extent of disease. The salivary and serum association of CRP shows a positive correlation, and it helps to include saliva as a noninvasive body fluid to measure the biomarkers. The salivary sample is easy to collect from children noninvasively, repetitive sample collection from the patients or participants to measure the CRP for early detection or to know the prognosis and therapy of the disease condition. The finding of risk factors over time reports salivary CRP as an essential biomarker to measure T2D screening.

Keywords

Biomarkers · Noninvasive · Saliva · Diagnostic fluid · C-reactive protein · Pathogenesis · Type 2-diabetes · Cardiovascular disease · Inflammation · Multiplex analyzer

Abbreviations

1,5-AG	1,5-anhydroglucitol
A2MG	α-2-macroglobulin
AD	Alzheimer's disease
ADA	American Diabetes Association
AMD	Age-related macular degeneration
AUC	Area under the curve
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
FDA-NIH	Food and Drug Administration-National Institutes of Health
GDF15	Growth differentiation factor 15
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
IFG	Impaired fasting glucose
IGFBP-2	Insulin-like growth factor binding protein-2
IL-1RA	Interleukin receptor-1 antagonist
IL-6	Interleukin-6
MI	Myocardial infarction
NW	Normal weight
OW/OB	Overweight/obese
PD	Parkinson's disease
PE	Phycoerythrin
ROC	Receiver-operating characteristics
SLE	Systemic lupus erythematosus

T2D	Type 2-diabetes
TMB	Tetramethylbenzidine
TNF-αR2	Tumor necrosis factor-α receptor 2

Introduction

Biomarkers have been used in diagnosing clinical diseases for several decades. The definition of "biomarker" is established in 2016 by the Food and Drug Administration-National Institutes of Health (FDA-NIH)-collaborative working group. Biological markers are the element quantified in a biological system as a marker of normal biological activity, pathogenic exposure, and its effect on a therapeutic intervention (Biomarkers Definitions Working Group 2001; Califf 2018). The biomarkers are measured from the molecular contents, histological observations, radiographic methods, or physiological characteristics (Biomarkers Definitions Working Group 2001). They are classified based on their applications. Diagnostics markers are used to identify the right disease by its measurements in any samples. The second is monitoring biomarkers measured with intermittent time points on the same patient to find the status of the disease. Biomarkers play a vital role in clinical medicine to monitor the extent of disease. Pharmacodynamic/response biomarkers are known as the biomarkers that change due to intervention or treatment or an environmental agent, and it is used in clinical patients and therapeutic process. The other biomarker that is present or shows the change in an individual or group during exposure to a medical outcome is a predictive biomarker. These markers are essential in the study design and operation of clinical trials. The prognostic biomarkers are used to measure the possibility of any clinical disease, occurrence, or progress of a medical condition. Prognostic biomarkers differ from predictive markers, which tends to find the effect of intervention in the patients. The biomarkers measured before or after an intervention or environmental exposure are known as safety biomarkers (Califf 2018; Chen et al. 2011).

Diabetes is one of the chronic metabolic diseases that the world is trying to control. Type 1 and type 2 diabetes developed due to the destruction of the pancreas by autoantibodies and through insulin resistance, respectively. It is one of the major health problems, and it is essential to provide its diagnosis and management. This chapter will discuss the salivary c-reactive protein for the diagnosis of diabetes.

Biomarkers of Type 2 Diabetes

The increase in diabetes prevalence requires more diagnostic markers to identify the disease and its extent of complications. The well-known biomarker for diabetes is glucose, insulin, and hemoglobin A1c (HbA1c). The advancement in omics technologies developed a new range of molecules, including genetic variants, RNA

transcripts, proteins, and small metabolites that act as biomarkers for diabetes and other diseases (Srinivasan et al. 2015). The clinical trials on type 2-diabetes (T2D) have shown their importance on biomarkers to determine the drug's or therapy's efficacy. Even though numerous biomarkers are available, spotting new biomarkers for T2D and its associated disease is difficult due to its heterogeneous nature. Several inflammatory, endothelial-specific biomarkers and markers originating from the microbiome have recently been identified for diabetic-related problems (Ahluwalia et al. 2019). Thorand et al. measured 47 biomarkers from nonfasting venous blood samples and selected six biomarkers in a panel which includes interleukin receptor-1 antagonist (IL-1RA), insulin-like growth factor-binding protein-2 (IGFBP-2), high-density lipoprotein (HDL) cholesterol, decorin, soluble E-selectin, and adiponectin that improves the prediction of T2D in the Monitoring of Trends and Determinants in Cardiovascular Disease/Cooperative Research in the Region of Augsburg (MONICA/KORA) (Thorand et al. 2021). Netrin-1, the anti-inflammatory protein, was significantly greater in impaired fasting glucose (IFG) and T2D serum samples than normal subjects by enzyme-linked immunosorbent assay (ELISA). Netrin was significantly positively correlated with fasting glucose, HbA1c, and insulin resistance index (Yim et al. 2018). Several studies have reported an increased expression of growth differentiation factor 15 (GDF15) in circulation and multiple tissues of prediabetes/T2D patients. GDF15 is an essential biomarker used for the early identification of metabolic disease (Berezin and Berezin 2020; Dominguez-Rodriguez et al. 2014; Lu et al. 2019; Shin et al. 2016).

The prospective study was conducted by Hu et al., on 32,826 normal women tested for inflammatory markers; later, 737 women developed diabetes and showed an increase in tumor necrosis factor-α receptor 2 (TNF-αR2), interleukin-6 (IL-6), and C-reactive protein (CRP) among diabetes-developed women compared to control subjects. This study reported CRP as the strong independent predictor of T2D (Hu et al. 2004). Irisin, a circulating myokine secreted by muscle, was measured on T2D patients exhibiting lowered expression by ELISA (Liu et al. 2013), and contrary to this report, the plasma levels in T2D patients showed an increased amount of plasma irisin and a positive association with E-selectin (Rana et al. 2017). A recent review compared six inflammatory markers, namely adiponectin, leptin, CRP, IL-6, TNF-α, and 1,5-anhydroglucitol (1,5-AG) in the saliva and serum. Adiponectin level was decreased in T2D, and contrarily, rest of the five proteins were increased in the T2D (Desai et al. 2020). American diabetes association (ADA) made HbA1c a diagnostic tool for prediabetes and diabetes patients in 2010. Recent evidence indicates that a specific profile of circulating miRNA that acts as an autocrine and endocrine regulator of gene expression could become an important biomarker to measure prediabetes and T2D. The circulating miRNA is derived from the cell-cell-communicating extracellular vesicle circulating in the human plasma. The miRNA used as a biomarker for T2D is linked to the development and progression of T2D, glucose metabolism, and insulin singling (He et al. 2007; Jimenez-Lucena et al. 2018; Karolina et al. 2011; Tavintharan et al. 2009).

C-Reactive Protein and Pathogenesis

C-reactive protein was first discovered in 1930 from blood samples of pneumococcal pneumonia patients and named as fraction C. CRP is secreted from the liver with response to a variety of inflammatory reactions and enters the bloodstream (Du Clos 2000; McCarty 1982). It is also synthesized in smooth muscle cells, lymphocytes, macrophages, endothelial cells, and adipocytes (Sproston and Ashworth 2018). CRP level increases during inflammatory conditions such as rheumatoid arthritis an autoimmune disease, cardiovascular disorder, and infection (Du Clos and Mold 2004; Sproston and Ashworth 2018). During acute phase inflammation, CRP level increases 25% in the plasma (Gabay and Kushner 1999; Morley and Kushner 1982) and increased up to 1000-fold in the serum with bacterial infection or tissue injury (Morley and Kushner 1982). The case-control study found the relationship between CRP, IL-6, and the autoimmune disease systemic lupus erythematosus (SLE) on patients with the active and inactive disease showed a significant increase in hsCRP than in patients with the active and inactive disease control. The study suggested a good correlation of hsCRP and IL-6 with the SLE disease activity index (Umare et al. 2017). Within 24–72 h of acute tissue damage, the CRP levels drastically increase from 1 μg/ml to more than 500 μg/ml in the conditions like trauma and cancer (Ciubotaru et al. 2005).

The relationship between vascular disease and inflammation is well documented. A marked level of CRP has been released during endothelial dysfunction. Koenig et al. conducted the MONICA Augsburg Cohort Study on 936 healthy men with an age range from 45 to 64 years; samples collected and followed for 8 years showed a positive correlation between CRP levels and the incidence of coronary heart disease (CHD) in the study population (Koenig et al. 1999). Myocardial infarction (MI) occurs in some patients even without an increase in cholesterol levels. In this case, the inflammatory marker hs-CRP is a risk indicator for MI. Previous findings prove that CRP acts as a stronger predictor of LDL cholesterol in healthy subjects. In this scenario, CRP can behave as the screening molecule to prevent hyperlipidemia and cardiovascular disease (CVD) (Ridker 2003). Numerous studies discussed in the review show CRP, the interactive marker, has a positive association with heart attack, stroke, metabolic syndrome, cigarette smoke, depression, and family history of premature CHD (Karakas and Koenig 2009).

Further, an examination of tobacco smoke exposure on the healthy youth population reports the increased salivary CRP expression, which correlates with active and passive smoking status (Azar and Richard 2011). Age-related macular degeneration (AMD) is an important cause of visual impairment. Several clinical studies results report the association of serum CRP and visual disease related to AMD. The patients with AMD showed a positive correlation of CRP and cholesterol levels, LDL, and non-HDL. The results describe the elevated levels of CRP in AMD patients (Colak et al. 2012; Schaumberg et al. 2007; Seddon et al. 2004).

Meta-analysis of ten different cross-sectional studies did not show any significant difference in the serum CRP levels of Alzheimer's disease (AD) compared to normal control. At the same time, mild and moderate AD reported lower serum CRP levels

than the control population. The diagnostic value of CRP can be used as a biomarker for mild and moderate AD (Gong et al. 2016; Luan and Yao 2018). Moreover, CRP was increased in ischemic infarction but not in hemorrhagic stroke. There is more evidence warranted to confirm CRP and hemorrhagic stroke (Roudbary et al. 2011). The neurodegenerative disorder Parkinson's disease (PD) has elevated levels of CRP and correlated with increased risk of PD in a cross-sectional study (Sawada et al. 2015). Patients infected with the current pandemic SARS-CoV2 showed high levels of CRP in their blood. CRP increase in the early stage of infection was associated with a lung infection and the seriousness of the disease (Mosquera-Sulbaran et al. 2021).

Type 2 Diabetes and CRP

T2D is one of the most important metabolic diseases associated with high morbidity and mortality worldwide. T2D patients show an increased level of CRP, TNF-α, and IL-6, which activate insulin-signaling pathways resulting in insulin resistance (Phosat et al. 2017). HbA1c is glycated hemoglobin, a marker of diabetes positively correlated with an increased level of CRP in elderly patients (de Rekeneire et al. 2006; Gorska-Ciebiada et al. 2015). A cross-sectional study of Hisayama residents aged 40–79 years, who exhibit a progressive increase in CRP as fasting glucose levels increased, observed a positive correlation between CRP and prediabetic glucose (Doi et al. 2005). Another noninstitutionalized US adult's data from National Health and Nutrition Examination Survey participants exhibited a higher percentage of increased CRP and correlated with increased HbA1c (King et al. 2003). Svensson et al. conducted a nationwide Danish Centre for Strategic Research on the newly diagnosed T2D patients and showed increased CRP levels in 40% of the study patients. Among the 40% CRP-elevated patients, more women (46%) than men (34%) were reported (Svensson et al. 2014). A retrospective cross-sectional study conducted in large Korean population results showed higher mean CRP levels with comorbidities and associated with increased prevalence of hypertriglyceridemia, diabetes, and metabolic syndrome after adjusting for socioeconomic and lifestyle characteristics in the multivariable analysis (Jeong et al. 2019). The prospective observational cohort study was conducted on Rio de Janeiro Type 2 diabetes patients; the CRP level in baseline and follow-up contributed to the cardiovascular risk prediction in patients with T2D (Cardoso et al. 2016). A recent study on Japanese subjects with differing glycemic statuses revealed the association of CRP with prediabetes and elevated levels of HbA1c (Kato et al. 2019).

Human Saliva as Diagnostic Fluid for Biomarkers

Different biological samples or biofluids can be used for biomarker discovery. The biofluids include plasma, serum, urine, saliva, cerebrospinal liquid, tears, amniotic

fluid, and body tissue or cell extracts. Readily available biofluids are plasma, serum, urine, and saliva for diagnostic purposes. Saliva and urine have grasped more attention in biomarker discoveries in recent years due to simple sample collection, noninvasiveness, cost-effectiveness, and can be easily repeated if samples are required (De Bock et al. 2010). Saliva has been used as a noninvasive and comparatively stress-free diagnostic fluid against blood. It is the ultrafiltrate of plasma containing proteins from blood or secreted from the salivary gland (Yoshizawa et al. 2013). Saliva contains many materials such as antibodies, microbes, DNA, RNA, lipid, metabolites, and proteins that help diagnose human diseases like cancers, diabetes, obesity, and autoimmune disease. The T2D-salivary proteome consists of a differentially abundant protein related to pathways regulating metabolic (42%), immune response (11%), and the remaining percentage consisting of the rest of the functions helps detect potential biomarkers of T2D (Rao et al. 2009). Inflammation occurred by several disease conditions including obesity, oxidative stress, rheumatoid arthritis, and autoimmune disease. Most of the studies showed the commonly explored salivary marker is CRP and antioxidant status. Table 1 discusses the biomarkers present in saliva and its measurement during different disease conditions.

Salivary Biomarkers in Type 2 Diabetes

Identification of Diabetes-Associated Serum Proteins Found in Saliva

In T2D, 214 proteins are reported as the serum-/plasma-circulating biomarkers. Among these, 130 biomarkers are identified and measured in saliva. Glucose, insulin, cortisol, adipokines, and inflammatory biomarkers were primarily analyzed in the salivary samples of T2D patients. While screening the 130 salivary biomarkers, 62 have been identified in diabetes patients' saliva (Srinivasan et al. 2015). Ladgotra et al. showed a positive correlation of diabetes markers such as glucose and amylase in both serum- and saliva-biological fluid of diabetes patients compared to healthy subjects (Ladgotra et al. 2016). Guo et al. conducted a study with iron disorder and diabetes on 88 patients and showed that serum and unstimulated salivary ferritin and hepcidin levels increased in T2D than in control groups. Both salivary and serum ferritin and hepcidin showed a positive correlation. Salivary ferritin levels were equivalent to the body's iron concentration (Guo et al. 2018). The α-2-macroglobulin (A2MG) is one of the several diabetic markers found in serum and saliva. A2MG was measured in control, and diabetic patients' salivary sample showed an elevated level of A2MG in T2D. A positive correlation between A2MG and HbA1c was observed, and this study suggests that A2MG is an indicator of glycemic control in T2D patients (Aitken et al. 2015). The type 1 and 2 diabetes subjects' saliva samples were divided into five groups based on A1C levels. The global salivary proteomic analysis demonstrates the proteomic profile changes observed in the saliva associated with A1C levels (Bencharit et al.

Table 1 Salivary biomarkers and its observation in different disease

Disease	Biomarker	Observation/regulation	References
Chronic periodontitis/ type 2 diabetes	Neopterin	Neopterin decreased in the study group	Fenol et al. (2017)
Type 2 diabetes	IL-6, CRP, and TNF-α	IL-6, CRP, and TNF-α higher than control participants	Agho et al. (2021)
Metabolic syndrome	Adiponectin and leptin	No difference between healthy subjects and metabolic syndrome patient	Thanakun et al. (2013)
Obesity and type 2 diabetes	CRP, nitric oxide, IL-1β, and glucose	The biomarkers did not show any significant difference compared to normal weight group	Janem et al. (2017)
Obesity	Interleukins, MCP-1, TNF-α, VEGF, MPO, MMP-9, adiponectin, resistin, and CRP	Salivary CRP, insulin, and leptin are found to be higher in obese children whereas adiponectin is decreased compared to normal weight children	Goodson et al. (2014)
Inflammatory arthritis	CRP	Increased CRP observed in arthritis condition compared to normal	Smith et al. (2018)
Myocardial infarction	Adiponectin and CRP	Increased CRP and decreased adiponectin observed in myocardial infarction patients with oral health and obesity	Ebersole et al. (2017)
Healthy participant with pain	NGF, CGRP, and BDNF	Measurable level of NGF, CGRP, and BDNF found	Jasim et al. (2018)
Metabolic syndrome with periodontitis	TNF-α and IL-10	TNF-α increased significantly in metabolic syndrome (MS) with chronic periodontitis (PD) or only MS or PD groups than control groups. IL-10 was significantly decreased in all groups compared to control participants	Chauhan et al. (2016)
Stress	CRP	African-American participated in a laboratory-based social-evaluative stressor task increased in response to the stressor task	Goetz and Lucas (2020)

IL interleukin, *CRP* C-reactive protein, *TNF-α* tumor necrosis factor alpha, *MCP-1* monocyte chemoattractant protein-1, *VEGF* vascular endothelial growth factor, *MPO* myeloperoxidase protein, *MMP-9* matrix metalloproteinase-9, *MS* metabolic syndrome, *PD* periodontitis, *NGF* nerve growth factor, *CGRP* calcitonin gene-related peptide, and *BDNF* brain-derived neurotropic factor

2013). The comparative investigation of salivary and serum proteins showed elevated ghrelin, α-amylase, and glucose in diabetic compared to normoglycemic subjects (Aydin 2007; Gupta et al. 2015).

Measurement of Diabetes-Related Serum Protein Markers in Saliva of Diabetic Patients

Salivary glucose in healthy subjects and newly diagnosed T2D patients was positively associated with serum glucose and HbA1c (Gupta et al. 2015; Mealey and Rose 2008). A systemic review conducted on the salivary glucose measurement reported that glucose increased in T2D patients' saliva. It correlates with HbA1c values, suggesting that salivary glucose measurement can be used as a potential biomarker for diabetes (Mascarenhas et al. 2014). Measurement of insulin in young lean and overweight/obese participants after low and high carbohydrate postprandial showed a significant correlation with plasma and saliva insulin levels. This finding could be used to measure insulin in saliva samples as a noninvasive method (Myette-Cote et al. 2017). Fabre et al. exhibited a significant correlation of salivary and serum insulin ($r = 0.92$; $p < 0.001$) in boys and girls of children aged 6–14 years. However, the samples stored at −20 °C for 7 days decreased 29.8% salivary insulin levels in the preserved specimens (Fabre et al. 2012). Cortisol, the glucocorticoid hormone, is one of the essential salivary stress biomarkers. The study on overweight Latino youth at risk for type 2 diabetes found that cortisol levels had a negative effect on insulin sensitivity. In addition, cortisol shows a negative correlation with insulin resistance in the study participants' saliva (Adam et al. 2010). The longitudinal multiethnic atherosclerosis study shows black, Hispanic, and non-Hispanic white populations demonstrated association of salivary cortisol with plasma IL-6, IL-10, and TNF-α (DeSantis et al. 2012). Adipose tissues secret pro and anti-inflammatory factors, including adipokines, cytokines, and chemokines. These adipocytes are involved in insulin resistance, inflammation, and adipogenesis. Several studies demonstrate the salivary measurement of adipokines and their association with serum levels (Mamali et al. 2012; Thanakun et al. 2013; Yin et al. 2012). The advantage and disadvantages of saliva for measuring biomarkers are discussed in Table 2.

Correlation of the CRP Levels in Saliva and Serum

Several epidemiological studies focused on the importance of CRP in finding the risk of T2D in serum and saliva. CRP is an important biomarker of systemic inflammation, an independent risk factor for T2D and CVD (Desai et al. 2020; Laakso 2019; Pay and Shaw 2019). Childhood obesity increases the incidence of T2D in the adult population. In the cross-sectional study conducted on black South African children with a mean age of 9.41 ± 1.55 years (70 Males, 100 Females), the salivary CRP levels measured by the ELISA kit showed a significant increase in obese children 7.31 ± 0.93 pg/ml ($n = 53$) compared to normal weight (NW) 6.77 ± 0.92 pg/ml ($n = 93$). In addition, obese children showed a higher salivary CRP section rate of 7.25 ± 0.99 pg/ml than normal weight 6.68 ± 0.98 pg/ml. The further finding revealed that poor cardiorespiratory fitness was associated with increased CRP secretion rate of saliva in overweight/obese (OW/OB) children (Naidoo et al. 2012). The editorial by Goodson and Welty discussed the salivary CRP as

Table 2 Advantages and disadvantages of saliva as a noninvasive diagnostic fluid for detection of biomarkers

Advantages	Disadvantages
• Noninvasive and cost-effective compared to blood samples • Sample collection is undemanding and does not require trained personnel to collect. It can be done by anyone and includes self-collection • Easy to collect a repeated sample on the same subject • Risk of exposure to deadly pathogens is less in saliva samples (for example, saliva contains factors that inhibit the infectivity of human immunodeficiency virus (HIV), thus very low risk of oral transmission) • There is no preprocessing technique required. Easy to ship and store	• Saliva sample collection methods are not standardized • The salivary flow rate may vary from sample to sample that affects the analytes present in it • Less saliva samples were collected. The presence of proteases may affect some biomarker detection • Some of the analytes present in the blood are significantly diminished or not reflected in saliva samples • If infected with the periodontal lesion, the sample is contaminated with blood cells

biomarkers used to identify the children at the risk of T2D (Goodson and Welty 2014). Goodson et al. used a noninvasive method to measure the metabolic disease risk factor in children and found six times higher CRP, three times more elevated insulin, and leptin in OB children compared to NW children (Goodson et al. 2014). The Luminex magnetic multiplex measurement of seven analytes, including CRP on NW and OW/OB children, reported increased expression of salivary CRP and a positive association with anthropometric measurement. The receiver-operating characteristics (ROC) curve for CRP is the superiority area under the curve (AUC) compared to other obesity markers (Selvaraju et al. 2019).

The results of the cross-sectional study on the human salivary CRP in healthy, type 1, and type 2 diabetes adult patients showed a significant number of T2D patients with $\geq$6 mg/l CRP levels. The detection of high saliva CRP shows the low-grade inflammatory process in T2D patients (Dezayee and Al-Nimer 2016). The ELISA measurement of saliva and plasma reports that hypertension patients showed significantly elevated CRP and glucose levels compared to nonhypertensive patients. The results show 2.6% of hypertension patients are reported to have diabetes compared to nonhypertensive (Labat et al. 2013). Evaluation of salivary CRP in T2D patients with periodontitis was low in nondiabetic and increased in T2D patients (Abbas and Jubouri 2013). A recent study on the noninvasive diagnosis of salivary CRP and other inflammatory markers in 39 T2D and 36 healthy controls. CRP was higher in diabetic (0.05 ± 0.04 μg/ml) than in healthy controls (0.02 ± 0.02 μg/ml) and was positively correlated with serum HbA1c levels (Agho et al. 2021). The diabetic patients with periodontal disease expressed elevated serum Hs-CRP and salivary IgA. The results show periodontal index is associated with poor glycemic control (Nazdar Mohammed et al. 2021). Table 3 summarizes the salivary CRP and its role in diabetes.

Table 3 Salivary CRP and its role in diabetes

Type of diabetes	CRP levels	Participants	References
Type 2 diabetes	Means CRP levels increased compared to healthy controls	Healthy controls ($n = 36$) and T2D ($n = 39$) participants	Agho et al. (2021)
Type 2 diabetes and type 1 diabetes	CRP levels positive (6 mg/l) in 36% T1D and 56% T2D	Healthy subjects ($n = 25$), T1D ($n = 25$), and T2D ($n = 50$)	Dezayee and Al-Nimer (2016)
Type 2 diabetes	Salivary CRP decreased in obese with T2D compared to normal weight and obese children	Normal weight ($n = 37$), obese ($n = 14$), and T2D ($n = 16$) children	Janem et al. (2017)
Type 2 diabetes	CRP levels in diabetes and periodontitis increased with respect to healthy and chronic periodontitis patients	Healthy participant, chronic periodontitis, and periodontitis with diabetes grouped as 20 patients each	Dholey et al. (2017)
Type 1 diabetes	Salivary CRP and homocysteine did not show any significant difference between patients and controls	Healthy participant and T1D ($n = 41$) in each group participated	Jazaeri et al. (2020)
Type 2 diabetes	Statistically significant CRP and a-amylase activity in diabetics than nondiabetic subjects	Control or nondiabetic ($n = 40$) and diabetic ($n = 173$) participated in the study	Omamuzo et al. (2021)
Type 2 diabetes	CRP levels impact the development of T2D compared to women with periodontitis	Women with gestational diabetes ($n = 90$) were followed 3 years ($n = 49$) for development of diabetes	Lima et al. (2017)

T2D type 2 diabetes, *T1D* type 1 diabetes, and *CRP* C-reactive protein

Diagnostic Methods of Salivary Biomarker Identification

The proteomics analysis found more than 2000 different salivary proteins and peptides present in saliva. Saliva chemistry is used to diagnose and measure oral and systemic clinical parameters and therapy monitoring. Some of the methods used to detect salivary biomarkers and metabolites are discussed in Table 4.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA detects the salivary biomarkers based on immunological analysis by a sandwich method in which the analyte is bound between capture and detection antibody. In brief, the saliva samples are centrifuged to remove the food particles and debris present in them. The antibody-coated ELISA plates were incubated with saliva samples and immobilized the analyte in the plate. Washing the plate removes the rest of the components present in saliva and incubated them with a secondary antibody with enzyme conjugate. This is followed by the incubation of the plate with the tetramethylbenzidine (TMB) substrate, the addition of the stop solution, and

Table 4 Salivary biomarkers measurement by different methodologies

Methodology	Biomarkers investigated	References
Enzyme-linked immunosorbent assay (ELISA)	Interleukins (IL-6 and IL-8) Common salivary protein	Franco-Martínez et al. (2020) and Wang et al. (2016)
Immunosensor/biosensor for multiplexed measurement or continuous monitoring	Hormones, antibodies, and biomarker related to disease	Malon et al. (2014)
Lab-on-a-chip (LOC)	Capture and quantify multiple analytes especially soluble analytes present in saliva	Steigmann et al. (2020)
Digital PCR or qPCR	Copy number variants measurement for different disease conditions	Venkatapoorna et al. (2019) and Zhang et al. (2009)
Luminex magnetics multiplex assay by Luminex xMAP technology	Quantitative measurement of salivary obesity, cardiovascular disease, and inflammatory markers (e.g., CRP, resistin, CCL2/MCP-1, TNF-α, IL-6, complement factor-D, and IL-10)	Goodson et al. (2014) and Selvaraju et al. (2019)
Proteomic analysis by two-dimensional gel electrophoresis and LC-MS-MS	More than 2000 protein markers associated with obesity, diabetes, and other diseases	Bencharit et al. (2013), Katsani and Sakellari (2019), Masood et al. (2018), and Pappa et al. (2018)

ELISA enzyme-linked sorbent assay, *LOC* lab-on-a-chip, *IL* interleukins, *CRP* C-reactive protein, *TNF-α* tumor necrosis factor-α, *qPCR* quantitative polymerase chain reaction, and *LC-MS-MS* liquid chromatography with tandem mass spectrometry

measuring the absorbance using a microplate reader. Salivary ELISA was conducted to measure the T2D markers, including CRP (Azar and Richard 2011; Guo et al. 2018; Labat et al. 2013; Yoshizawa et al. 2013).

Multiplex Analysis

Multiplex immunoassay/multiplex magnetic assay is used to measure the biomarkers present in the saliva samples of different diseases, including T2D (Fig. 1). Centrifuged saliva was incubated with a microparticle cocktail containing different biomarker capture antibodies coated with unique infrared dye-magnetic beads. The biomarkers bind to the specific capture antibodies, incubated with biotinylated detection antibodies specific to biomarkers of interest. It forms an antigen-antibody complex incubated with phycoerythrin (PE)-conjugated streptavidin. The beads are washed and detected using a multiplex analyzer that contains dual-laser flow-based instruments. One laser detects the beads, and the second laser detects analytes-conjugated PE-derived signal and quantified based on standard curve analyzed

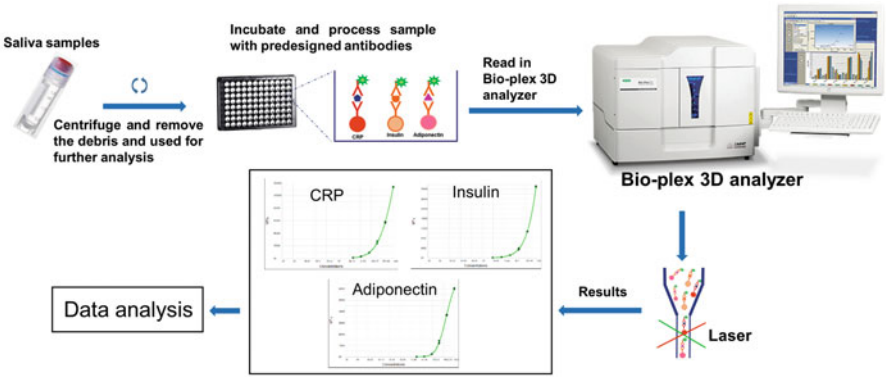

Fig. 1 The figure shows the diagrammatic representation of salivary analyte analysis by Multiplex analyzer. Luminex xMAP technology is used to measure a greater number of analytes in a smaller volume of samples. The two laser beams help to detect the bead used and the analytes measured

simultaneously. Several studies have used multiplex analysis to measure the salivary biomarkers (Arellano-Garcia et al. 2008; Selvaraju et al. 2019; Williamson et al. 2012).

Other Detection Methods

- **Biosensor and chip analysis:** The development of lab-on-a-chip is an electronic taste chip method. The wearable biosensor array platforms develop chemical and immunological reactions that capture single analytes in saliva and transfer the data (Christodoulides et al. 2005; Steigmann et al. 2020).
- **Lab on paper:** Existing diagnostic methods required sophisticated laboratory instruments and expensive reagents. Hence, cellulose and flexible transparency paper-based analytical devices detect salivary biomarkers and provide promising disease diagnosis and prevention solutions (de Castro et al. 2019; Sher et al. 2017).
- **Nanoproteomics:** Detection of low abundance biomarkers from saliva is difficult, and it needs high-throughput technological advancement. Nanotechnology provides sensitivity and reduces detection time, and multiplexing novel methods. Nanoproteomics presents significantly less volume detection, less assay time with low sample consumption for biomarker detection (Ray et al. 2011).

Applications to Prognosis, Other Diseases, or Conditions

CRP an inflammatory marker has more application in clinical as a predictive marker of numerous diseases in recent years. CRP levels are upregulated in inflammatory disease, cardiovascular disease, rheumatic disease, diabetes, etc. and are used as an important biomarker for predicting the disease. Results from serum/plasma CRP of

patients are positively correlated with salivary CRP in a disease condition (Srinivasan et al. 2015). It is helpful to measure CRP in saliva to know the severity of diabetes and develop a reliable noninvasive biomarker. Salivary biomarkers are associated with several diseases such as pneumonia (Omran et al. 2018), systemic lupus erythematosus (Stanescu et al. 2018), asthma (Takemura et al. 2006), obesity markers (Selvaraju et al. 2019), cardiovascular disease (Ridker 2003), and diabetes (Phosat et al. 2017). New methods are developed to report the salivary CRP levels and their application to clinical studies.

Mini-dictionary of Terms

- **Biomarkers:** Biomarkers refer to body fluids that contain biological markers related to protein, protein fragments, metabolites, genomic materials, and cellular materials used to identify an earlier diagnosis of disease or progress of therapy.
- **Saliva:** Saliva is a hypotonic solution secreted from the salivary gland, and it is one of the essential body fluids for measuring biomarkers. Saliva constitutes glucose, nitrogen materials, lipid profile, proteins, hormones, amylase, enzymes, sodium, calcium, chloride, bicarbonate, and magnesium from serum. In addition, saliva contains more than 700 microorganisms.
- **C-reactive protein:** CRP is a pentameric protein secreted by the liver and has proinflammatory and inflammatory properties. Minor elevation of CRP is found in diabetes, moderate elevation in systemic inflammation and myocardial infarction, and marked and severe election in acute bacterial and viral infections.
- **Diabetes:** Diabetes shows increased blood glucose levels and is classified into two types. Type 1 diabetes does not synthesize insulin, and T2D is not utilizing or synthesizing insulin well in the body, thereby increasing blood glucose levels.
- **Noninvasive:** Noninvasive is one of the methods used to collect biological samples for diagnostic purposes. It does not require special equipment to collect biological materials.

Key Facts of C-Reactive Protein

- Diabetes is an essential metabolic disease that the world faces and is trying to control.
- Diabetes prevalence needs different diagnostic methods to find the markers to know the disease extent and early prevention.
- Saliva is one of the important body fluids which contains most of the serum contents and other biomarkers for diagnosis of disease.
- C-reactive protein synthesized from the liver during inflammatory or other disease conditions, and it is a circulating marker present in serum, saliva, and urine.
- Development of noninvasive methods gains more attention for early detection and repetitive sample collection from patients.

Summary Points

- The increasing number of diabetes patients need more new and efficiently measurable diagnostic markers to identify the disease.
- Few studies have reported that increased concentration of CRP is linked with future diabetes.
- The chapter discusses the different methodological descriptions used to measure salivary CRP.
- The salivary and serum positive correlation makes salivary CRP a noninvasive biomarker for identifying diabetes.
- Salivary CRPs are useful for repetitive sample collection on the same patient to know the prognosis and extent of therapy given to the patient.

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Assessing Insulin Sensitivity in People with Type 1 Diabetes Without Euglycemic-Hyperinsulinemic Clamps

16

Andrzej S. Januszewski and Alicia J. Jenkins

Contents

Introduction 351
Euglycemic-Hyperinsulinemic Clamp 352
Estimation of Insulin Sensitivity 353
Estimation of GDR in Type 1 Diabetes 357
Application to Prognosis and Other Diseases or Conditions 363
Mini-Dictionary of Terms 364
Key Facts 368
Summary Points 368
References 368

Abstract

The euglycemic-hyperinsulinemic clamp is the "gold standard" in assessing insulin sensitivity. Due to high demands on the subject, need for experienced and skilled operators, and high costs, it remains predominantly a research tool. Multiple mathematical formulae have been developed to estimate insulin sensitivity using various biomarkers. Most variables used reflect body habitus, blood pressure, HbA1c, and lipids. Although most show relatively good correlation with insulin sensitivity measures from the euglycemic-hyperinsulinemic clamp

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series they are derived from, relatively few are validated using independent clamp datasets. Associations with other factors, which are usually mechanistically related to insulin resistance, such as inflammation and adiposity (reflected by adipokine levels) are also of interest, as these as well as clinical markers may suggest therapeutic targets.

Keywords

Estimation · Glucose disposal rate · Insulin sensitivity · Equation

Abbreviations

BMI	Body mass index
DBP	Diastolic blood pressure
dur	Duration
e	Estimated
ffm	Free fat mass
FL	Fluorescence
G	Glucose concentration
GDR	Glucose disposal rate
GFR	Glomerular filtration rate
HbA1	Glycated hemoglobin
HbA1c	Glycated hemoglobin
HDL-C	High-density lipoprotein cholesterol
HOMA	Homeostatic model assessment
HT	Hypertension
I	Insulin concentration
ICAM	Intercellular cell adhesion molecule 1
IS	Insulin sensitivity
ISI	Insulin sensitivity index
ln	Natural logarithm
M	Metabolized glucose
MCR	Metabolic clearance rate
mM	mmol/L
OGTT	Oral glucose tolerance test
PP	Pulse pressure
QUICKI	Quantitative insulin sensitivity check index
RAGE	Receptor for advanced glycation end products
Si	Sensitivity index
T1D	Type 1 diabetes
TDD	Total daily dose of insulin
TG	Triglycerides
VAI	Visceral adiposity index
WC	Waist circumference
WH	Waist-to-height ratio
WHR	Waist-to-hip ratio

Introduction

While the incidence of type 1 diabetes varies widely by ethnicity, its prevalence is increasing in most countries, and this condition is associated with potential for acute and chronic complications (International Diabetes Federation 2021). As the risk of complications (hypoglycemia, micro- and macrovascular complications, and premature death) is increased by poor glycemic control and insulin resistance and costs dearly at both personal and socioeconomic levels, there is a growing interest in the assessment and treatment of low insulin sensitivity in people with type 1 diabetes (Szadkowska et al. 2008; Kilpatrick et al. 2007). The terms "insulin sensitivity" and its converse of "insulin resistance" are often used interchangeably. Even though insulin resistance is predominantly recognized in people with type 2 diabetes or pre-diabetes, there is also a wide spectrum of insulin sensitivity in people with type 1 diabetes (Szadkowska et al. 2008). As well as genetic factors, there are many other contributors to insulin resistance, including family history of type 2 diabetes; adiposity; sedentary lifestyle; growth spurts, including puberty; dysglycemia; dyslipidemia; inflammation; hyperinsulinism secondary to artificial delivery of insulin into the systemic circulation bypassing first-pass hepatic insulin clearance (Gregory et al. 2020); intercurrent illness; stress; and some drugs. This impaired response to exogenous insulin is common in in patients with type 1 diabetes and is sometimes called "double diabetes" (Teupe and Bergis 1991). Insulin sensitivity and action involves complex interplays between hepatic, peripheral, and adipose tissue metabolism and glucose utilization (Donga et al. 2015).

The euglycemic-hyperinsulinemic clamp (or hyperinsulinemic-euglycemic clamp) developed by DeFronzo in the late 1970s remains a "gold standard" for the assessment of insulin sensitivity in humans (Defronzo et al. 1979). A crucial aspect for the success of the clamp-derived measurements is the maintenance of steady-state conditions, defined as a period of at least 30 min, during which the coefficients of variation (standard deviation divided by mean) for blood glucose, plasma insulin, and glucose infusion rate are less than 5% (Katz et al. 2000; Singal et al. 2010). Limitations of the clamp technique are labor-intensiveness, cost, and need for experienced staff and specialized equipment; thus, clamp studies remain predominantly a research tool. The classic clamp studies quantify whole-body insulin resistance (Donga et al. 2015), but more recent research techniques, including isotope dilution (Dabelea et al. 2011; Zhang et al. 2018), enable the measurement of insulin sensitivity in the liver, skeletal muscle, and adipose tissue (Snaith et al. 2021). To facilitate the assessment of insulin sensitivity/resistance in clinical research and potentially in clinical practice, relatively simple formulae mainly based on clinically available data have been developed.

We will now overview the euglycemic-hyperinsulinemic clamp principles and outputs and summarize existent formulae for estimating insulin resistance in clinical research and clinical practice. As well as formulae developed for use in type 1 diabetes, for completeness, we will also include formulae developed from type 2 diabetic and non-diabetic subjects.

Euglycemic-Hyperinsulinemic Clamp

The goal of the euglycemic-hyperinsulinemic clamp is to increase circulating insulin concentration (to approx. 100 μU/mL) by a continuous intravenous infusion of short- or rapid-acting human insulin and to maintain it at that level, usually for 120–180 mins. To avoid hypoglycemia, the plasma glucose concentration is maintained at euglycemic level through 20% dextrose infusion at variable rate using a negative feedback principle (Defronzo et al. 1979). The rate of dextrose infusion is guided by frequent (every 5–10 min) bedside measures of glucose and calculation of the require insulin infusion rate. At the plasma glucose steady state, the amount of infused glucose is equal to the amount of glucose uptake by the body (insulin sensitivity). The steady state is usually maintained for at least 30 min., but it is not unusual to keep it for 90 min. Maintaining euglycemia and hyperinsulinemia prevents endogenous glucose production.

The following parameters can be derived from the clamp:

M – glucose metabolized. Once euglycemia is maintained (steady state), the amount of infused glucose (to maintain euglycemia during the clamp) is equal to the amount of glucose being metabolized in the periphery (Defronzo et al. 1979).

I – insulin concentration. This reflects the amount of infused insulin required to maintain hyperinsulinemia and allowing to reach euglycemic steady state (Defronzo et al. 1979). It is usually measured during the last 30 or 60 min of a 2-h clamp.

M/I ratio – a measure of the amount of glucose metabolized per unit of plasma insulin concentration. For convenience of data expression, M/I can be presented either as a result of multiplying "raw" M/I by 100 or by taking log 10 value of it.

GDR – glucose disposal rate (mg/kg body weight/min) which is calculated using the formula:

$$GDR = \text{Glucose infusion rate} * 180 * \text{weight} - (G_{T1} - G_{T0}) * 0.095 \quad (1)$$

In this formula, the glucose infusion rate is expressed in mM/kg/min, G is plasma glucose concentration (mM), *T*0 and *T*1 are arbitrary set intervals, 180 is a factor to convert glucose levels from mM to mg/dL, and weight is measured in kg. This measured GDR is a result of normalization of the *M* parameter to the subject's body weight (Patarrão et al. 2014).

MCR – metabolic clearance rate of glucose. Calculated by dividing glucose infusion rate by the increase in plasma glucose concentration above basal (Defronzo et al. 1979; Best et al. 1981). This parameter though is of very limited use, especially in clamps in people with type 1 diabetes (Best et al. 1981; Tajiri et al. 2011). MCR can also be calculated for insulin, using the same principles (Ahmad et al. 1994).

$$MCR_{\text{Glucose}} = \frac{\text{Glucose infusion rate}}{\text{Glucose concentration at steady} - \text{state}} \quad (2)$$

Estimation of Insulin Sensitivity

There are several clinical tools existing to estimate insulin sensitivity (Gutch et al. 2015; Patarrão et al. 2014; Soonthornpun et al. 2003). Not all are ideal for people with type 1 diabetes but will be presented here for completeness.

For example, the homeostatic model assessment (*HOMA*), a method for assessing insulin resistance from measurement of glucose and insulin (or C-peptide concentrations), is more suitable for people without diabetes or with pre-diabetes or type 2 diabetes, ideally not requiring exogenous insulin. First described in 1985, its appeal arises from the need for only a single blood sample to be assayed for insulin (or C-peptide) and glucose (Wallace et al. 2004). A HOMA Score can be calculated both for the beta cell (HOMA-Beta) and for insulin resistance (HOMA-IR). The main issue with using HOMA in type 1 diabetes is that it requires sample to be collected at steady-state glucose and insulin levels (i.e., ideally during a clamp), thus failing to provide an information about the results of insulin/glucose stimulation.

$$HOMA = \frac{\text{Fasting insulin} * \text{Fasting glucose}}{22.5} \tag{3}$$

Similar principle (and disadvantage) is applicable to quantitative insulin sensitivity check index (*QUICKI*) (Katz et al. 2000).

$$QUICKI = \frac{1}{\ln \text{Fasting insulin} + \ln \text{Fasting glucose}} \tag{4}$$

where insulin is measured in μIU/mL and glucose in mM (HOMA) or mg/dL (QUICKI).

Multiple insulin sensitivity indexes have been developed over recent years. They are mostly applicable in type 2 diabetes as a common feature is that they need for blood sample(s) to be collected during an oral glucose tolerance test (OGTT). OGTT is not necessary in the diagnosis of type 1 diabetes, and its value is being diminished in the past 20 years, even for type 2 diagnosis (Davidson 2002). However, OGTT can be used to exclude the diagnosis of diabetes when hyperglycemia or glycosuria is recognized in the absence of typical causes (e.g., intercurrent illness, steroid therapy) or when the patient's condition includes renal glucosuria (Lamb 2021). Insulin sensitivity indexes derived from OGTT metrics are:

– *Cederholm and Wibell index* (Cederholm and Wibell 1990):

$$ISI_{\text{Cederholm}} = \frac{75,000 + (G_0 - G_{120}) * 1.15 * 180 * 0.19 * \text{weight}}{120 * G_{\text{mean}} * \ln I_{\text{mean}}} \tag{5}$$

where 75,000 represents glucose load (mg) in OGTT and G is the plasma glucose concentration (mM) (0, fasting; 120, 2 h of OGTT; mean, average during OGTT); 1.15, factor transforming venous blood glucose to plasma values (only applicable if

glucose is not measured in plasma); 180, factor to convert glucose concentration from mM to mg/dL; 0.19, glucose space in liter per kg of body weight (measured in kg); and I, plasma insulin concentration.

– *Gutt et al. index* (Gutt et al. 2000), which is an adaptation of the above by omitting the constant terms and using the plasma glucose and insulin concentration from fasting (0 min) and 120 min samples from the OGTT:

$$ISI_{0,120} = \frac{75,000 + (G_0 - G_{120}) * 0.19 * \text{weight}}{120 * G_{\text{mean}} * \ln I_{\text{mean}}} \tag{6}$$

where glucose is measured in mg/dL and weight in kg.

– *Avignon et al. index* (Avignon et al. 1999). Those are three indexes derived from fasting state (i.e., before OGTT, 2 h of OGTT, and averaging both):

$$Sib = \frac{10^8}{I_0 * G_0 * VD} \tag{7}$$

$$Si2h = \frac{10^8}{I_{120} * G_{120} * VD} \tag{8}$$

$$SiM = \frac{(0.137 * Sib) + Si2h}{2} \tag{9}$$

where *b* denotes basal; *M* denotes mean; G and I are concentrations of glucose (mM) and insulin (mIU/L), respectively; and *VD* is the glucose distribution volume calculated as $VD = 150$ mL/kg of body weight (Bergman et al. 1987).

– *Matsuda et al. index* (Matsuda and Defronzo 1999):

$$ISI_{\text{Matsuda}} = \frac{10,000}{\sqrt{G_0 * I_0 * G_{\text{mean}} * I_{\text{mean}}}} \tag{10}$$

where *G* and *I* are concentrations of glucose (mM) and insulin (mIU/L), respectively (0, fasting; mean, average from 0 to 2 h of OGTT).

– *Belfiore et al. index* (Belfiore et al. 1998):

$$ISI_{\text{Belfiore}} = \frac{2}{(G_S/G_N) * (I_S/I_N) + 1} \tag{11}$$

where *G* and *I* are concentrations of glucose (mM) and insulin (mIU/L), respectively (*S*, subject values; *N*, normal reference values), expressed as fasting values or as areas obtained during OGTT at baseline and 2 h or at baseline, 1 h, and 2 h.

– *Stumvoll et al.* (2001) developed a series of indexes (insulin sensitivity and MCR, examples below) using stepwise linear regression analysis and included various clinical (OGTT sample results) and demographic parameters:

$$ISI_{\text{Stumvoll}} = 0.156 - 0.0000459 * I_{120} - 0.000321 * I_0 - 0.00541 * G_{120} \quad (12)$$

$$ISI_{\text{Stumvoll}} = 0.222 - 0.00333 * BMI - 0.0000779 * I_{120} - 0.000422 * \text{age} \quad (13)$$

$$MCR_{\text{Stumvoll}} = 13.273 - 0.00384 * I_{120} - 0.0232 * I_0 - 0.463 * G_{120} \quad (14)$$

$$MCR_{\text{Stumvoll}} = 19.24 - 0.281 * BMI - 0.00498 * I_{120} - 0.333 * G_{120} \quad (15)$$

where insulin concentration was measured in pM and glucose in mM.

– *McAuley et al.* (2001) developed an insulin sensitivity index applicable to assess insulin resistance in 178 normoglycemic adults (25–68 years of age) undergoing euglycemic-hyperinsulinemic clamp. Variables for an equation were selected using regression analysis with backward elimination and bootstrapped procedure with replacements. Data from measurements performed in the last 60 min of the clamp were used. Their index is reflecting M/I value (as continuous or categorical variable), with M being corrected for fat-free mass (Mffm) (Ferrannini et al. 1997). Mffm/I values during the clamp were ranging between 2.3 and 25.9 mg/kg/min and Mbw/I values (M being normalized by total body weight) 1.2–18.6 mg/kg/min. Two equations (for ISI as a continuous variable) are:

$$ln\left(\frac{M_{ffm}}{I}\right) = 3.29 - 0.25 \ln(I) - 0.22 \ln(BMI) - 0.28 \ln(TG) \quad (16)$$

$$ln\left(\frac{M_{ffm}}{I}\right) = 2.63 - 0.28 \ln(I) - 0.31 \ln(TG) \quad (17)$$

And two equations for calculation ISI as categorical variable (which can be transformed into logit form to get probability of having insulin resistance defined as ISI $\leq$ 6.3 M*mU^{-1}*L^{-1}) are:

$$ln\left(\frac{M_{ffm}}{I}\right) = -3.62 + 1.90 \ln(I) - 0.43 \ln(BMI) + 1.30 \ln(TG) \quad (18)$$

$$ln\left(\frac{M_{ffm}}{I}\right) = -4.93 + 1.81 \ln(I) + 1.24 \ln(TG) \quad (19)$$

where I is the insulin concentration (mU/L); BMI, body mass index (kg/m^2); and TG, triglyceride concentration (mM). The formula (17) has been shown to correlate with fasting insulin levels in type 2 diabetes (Kaur et al. 2021).

Other insulin sensitivity indexes which do not require a clamp study or an OGTT:

– *Insulin-to-glucose ratio* (Caro 1991) and *Bennett index* (Anderson et al. 1995) are indexes calculated from fasting insulin and glucose levels. The former is a simple division of fasting insulin level by fasting glucose level, whereas the latter is an inverse of the product of log transformation of fasting insulin and fasting glucose:

$$IS_{\text{Bennet}} = \frac{1}{ln\,G_0 * ln\,I_0} \tag{20}$$

These indexes have been showed to have ethnic differences in young non-diabetic women (Chen and Scholl 2002) and were used as one of the outcomes in randomized trial assessing effects of exercise on postprandial glucose levels (Wheeler et al. 2020).

Lipids are involved in insulin sensitivity with skeletal muscle triglyceride content correlating with insulin resistance (*Ma et al.* 2020)*, and HDL is involved in pancreatic insulin secretion (Fryirs et al.* 2010*). Circulating lipid levels are easier to obtain and have been used mainly in the general and type 2 diabetic population.*

TG-to-HDL-C ratio has been showed as a useful parameter associated with and predicting of insulin resistance (Kannel et al. 2008) and cardiometabolic risk (Kannel et al. 2008; Di Bonito et al. 2012). It has also shown ability to predict the development of type 2 diabetes (De Leon et al. 2012) and coronary heart disease (Hadaegh et al. 2009), being associated with cardiovascular events and all-cause mortality (Bittner et al. 2009) and being an independent risk factor in predicting chronic kidney disease in healthy adults (Ho et al. 2015).

$$\frac{TG}{HDL - C} \tag{21}$$

This index was also used as a dependent variable for the identification of insulin resistance using machine learning (Chakradar et al. 2021).

Visceral adiposity index (VAI) was developed in 2010 by Amato et al. (Amato et al. 2010) to assess visceral fat function associated with cardiometabolic risk. The authors analyzed the relationship between insulin sensitivity (expressed as M value), using data from clamps of 74 subjects (adults, 30 men, 44 women, 24 with type 1 diabetes, 29 with type 2 diabetes, and 21 with bob-alcoholic fatty liver disease and polycystic ovary syndrome), and VAI and have found that they correlated negatively with R-squared (this is not adjusted R-squared) of 0.52. Additionally, M value did not correlate with either waist circumference (WC) or BMI. They have presented two formulae, separate for men and women:

$$VAI_{\text{Males}} = \left(\frac{WC}{39.68 + (1.88 * BMI)}\right) * \left(\frac{TG}{1.03}\right) * \left(\frac{1.31}{HDL - C}\right) \tag{22}$$

$$VAI_{\text{Females}} = \left(\frac{WC}{36.58 + (1.89 * BMI)}\right) * \left(\frac{TG}{0.81}\right) * \left(\frac{1.52}{HDL - C}\right) \tag{23}$$

where *WC* was in cm and *TG* and *HDL-C* in mM. Later the same group showed a series of cut-off values for VAI associated with visceral adipose dysfunction and cardiometabolic risk (Amato et al. 2011). It has also been shown, in a separate validation of VAI, that cut-off value of >1.84 has 82% sensitivity and 71% specificity for the detection of the metabolic syndrome in adult patients with type 1 diabetes (Ferreira-Hermosillo et al. 2020).

Both *TG/HDL-C* and *VAI* have been shown to be able to assess insulin resistance in adults with type 1 diabetes (Uruska et al. 2018).

Estimation of GDR in Type 1 Diabetes

More commonly used in the literature for people with type 1 diabetes are measures estimating the glucose disposal rate (GDR). Ideally, these should derive from a group of type 1 diabetes patients and validated in a separate series of clamp studies using a similar clamp protocol.

In type 1 diabetes, glucose utilization/insulin sensitivity/resistance assessment is sometimes assessed by the simple measure of exogenous total daily dose (TDD) of insulin/kilogram body weight. But this value does not always correlate well with clamp study results (Wallace and Matthews 2002).

The letter "e" in the formulae below indicates that it is an estimation of the parameter calculated by the equation.

Several groups, including the authors, have derived formula(e) to calculate estimated glucose disposal rate (eGDR, sometimes also called glucose distribution rate) using a range of biomarkers (clinical, demographic, and laboratory).

The first and one of the most widely used formulae is the one developed by *Williams et al.* (2000) in 2000. They performed euglycemic-hyperinsulinemic clamps in 24 adults with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications (EDC) study and used multivariate regression analysis to find a set of variables to estimate GDR, which was measured in the last 30 min of the clamp. Patients were selected based on the probability of presenting various levels of insulin resistance based on the insulin resistance scoring system (hypertension (HT), waist-hip ratio (WHR), TG and HDL-C levels, and family history of type 2 diabetes) and eligibility criteria (age > 18 yrs., HbA1 < 11.4% (HbA1c <9.4%), serum creatinine <1.5 mg/dL, and normal hemoglobin and hematocrit). The clamp GDR was between 2 and 12 mg/kg/min. Using forward stepwise regression, they obtained a model containing WHR and HT (absence (0) or presence (1), defined, in a bit unorthodox way, as BP above 140/90 mmHg or usage of any anti-hypertensive drugs):

$$eGDR = 21.17 - 14.66 * WHR - 3.96 * HT \tag{24}$$

The model adjusted R-squared 0.54. The authors forced HbA1 (%) (please note it is not HbA1c) into the regression analysis and delivered the final model:

$$eGDR = 24.31 - 12.22 * WHR - 3.29 * HT - 0.57 * HbA1 \quad (25)$$

With adjusted R-squared of 0.57, i.e., 57% of variance of GDR observed in their clamps were explained by this model. The formula has been later adjusted to reflect usage of waist circumference (WC, cm) rather than waist-to-hip ratio (Epstein et al. 2013) and HbA1c (%) rather than HbA1 (Miller et al. 2019; Kilpatrick et al. 2007):

$$eGDR = 21.158 - 0.09 * WC - 3.407 * HT - 0.551 * HbA1c \quad (26)$$

$$eGDR = 24.395 - 12.971 * WHR - 3.388 * HT - 0.601 * HbA1c \quad (27)$$

This original manuscript to date has been cited over 300 times (Nov 2021). The original formula, although not validated in the independent dataset, has been associated (low eGDR) with the presence of chronic diabetes complications (Chillaron et al. 2009; Lam-Chung et al. 2021) and overall mortality (Olson et al. 2002). The original formula was also later updated to use BMI rather than WC or WHR and showed an association with dyslipidemia in type 1 diabetes (Nishtala et al. 2020). eGDR calculated from this formula was predictive of mortality in adults with type 1 diabetes (Nystrom et al. 2018):

$$eGDR = 19.02 - 0.22 * BMI - 3.26 * HT - 0.61 * HbA1c \quad (28)$$

The next eGDR formula was developed by *Dabelea et al.* in 2010 (Dabelea et al. 2011). The authors performed 85 euglycemic-hyperinsulinemic clamps in 60 youth (12–19 yrs. of age) with type 1 and 25 with type 2 with HbA1c <12%, serum creatinine <114 μM, and normal hemoglobin and hematocrit. Data from 53 youths (39 with type 1 and 14 with type 2) were used to develop a model, using multiple linear regression approach, estimating GDR. The data from the remaining 32 subjects (21, type 1; 11, type 2) were used for model cross-validation. Clamp GDR was between approx. 1 and 15 mg/kg/min in people with diabetes and was calculated in the last 30 min of the clamp. The best model consisting of WC (cm), HbA1c (%), and TG (mg/dL) resulted in adjusted R-squared of 0.74:

$$elnGDR = 4.647 - 0.02 * WC - 0.098 * HbA1c - 0.002 * TG \quad (29)$$

and most "practical" model (consisting of waist circumference only) resulted in adjusted R-squared of 0.59:

$$elnGDR = 3.734 - 0.022 * WC \quad (30)$$

Validation of the former model in clamp studies resulted in R-squared of 0.42 for youths with diabetes and 0.38 for youths without diabetes. The paper has been cited 80 times to date (Nov 2021). The Dabelea et al. formula has been associated with glycemic control and dyslipidemia in youth with type 1 (Amor et al. 2020) and type 2 diabetes (Brady et al. 2021). The calculated eGDR was significantly lower in type

2 diabetes than in type 1 diabetes subjects, and it did not correlate with the change of eGFR in youth with diabetes in the SEARCH study (Westreich et al. 2021) nor was it different among people with type 1 diabetes treated with insulin or insulin combined with metformin (Yang et al. 2020a).

Duca et al. in 2016 presented a few formulae derived from 58 people (26 with type 1 diabetes and 32 without diabetes) and validated them in 19 people (10 with type 1 diabetes and 9 without diabetes) participating in the Coronary Artery Calcification in Type 1 Diabetes (CACTI) cohort (Duca et al. 2016). Insulin sensitivity (IS), expressed in mg/kg fat-free mass/min, range can be estimated to be between 1 and 13 mg/kg ffm/min in type 1 diabetes subjects. All the parameters were calculated from the final 30 min. of the clamp.

Their best model (eIS) for adults with type 1 diabetes included (WC (cm), TDD per kg, adiponectin (μg/mL), TG (mM), and DBP (mmHg):

$$elnIS = 4.062 - 0.013 * WC - 1.096 * TDD + 0.02 * Adiponectin - 0.272 * TG - 0.007 * DBP \quad (31)$$

or if TG expressed in mg/dL:

$$elnIS = 4.062 - 0.013 * WC - 1.096 * TDD + 0.02 * Adiponectin - 0.003 * TG - 0.007 * DBP \quad (32)$$

This model resulted in an adjusted R-squared of 0.64. Validation of this model resulted in R-squared of 0.48 for people with type 1 diabetes. They have also suggested the model without adiponectin, with adjusted R-squared of 0.63.

$$elnIS = 4.108 - 0.013 * WC - 1.058 * TDD - 0.313 * TG - 0.008 * DBP \quad (33)$$

or if TG expressed in mg/dL:

$$elnIS = 4.108 - 0.013 * WC - 1.058 * TDD - 0.0035 * TG - 0.008 * DBP \quad (34)$$

The best model for individuals without diabetes included HbA1c (%) rather than WC and fasting glucose (G_0, mM) rather than TDD/kg:

$$elnIS = 7.472 - 0.013 * WC - 0.25 * HbA1c - 0.357 * G_0 + 0.019 * Adiponectin - 0.287 * TG - 0.006 * DBP \quad (35)$$

or with glucose expressed in mg/dL and TG in mg/dL:

$$elnIS = 7.472 - 0.013 * WC - 0.25 * HbA1c - 0.02 * G_0 + 0.019 * Adiponectin - 0.003 * TG - 0.006 * DBP \quad (36)$$

And the models without adiponectin:

$$elnIS = 7.191 + 0.102 * Sex - 0.014 * WC - 0.333 * HbA1c - 0.232 * G_0 - 0.279 * TG \quad (37)$$

or with glucose in mg/dL and TG in mg/dL:

$$elnIS = 7.191 + 0.102 * Sex - 0.014 * WC - 0.333 * HbA1c - 0.013 * G_0 - 0.316 * 10^{-2} * TG \quad (38)$$

In non-diabetic subjects, the adjusted R-squared for the model was 0.63 and in validation 0.59. This manuscript has been cited 21 times to date (Nov 2021). It has been shown that eIS calculated using this formula is negatively correlated with cardiovascular risk in type 1 diabetes (Cano et al. 2020; Jensen et al. 2019) and predicts micro- and macrovascular complications in adults with type 1 diabetes (Bjornstad et al. 2016).

In 2017, *Zheng et al.* (2017) presented a formula derived from 36 adults with type 1 diabetes (16 with childhood onset and 20 with type 1 diabetes diagnosed as adults) using stepwise linear regression. GDR measured during the final 30 min of the clamp ranged between 4.2 and 11.8 mg/kg/min. The best combination of clinical parameters was containing HbA1c (%), DBP (mmHg), and WHR:

$$elnGDR = 4.964 - 0.121 * HbA1c - 0.012 * DBP - 1.409 * WHR \quad (39)$$

Adjusted R-squared for this model was 0.62. Authors also showed that a simple model with HbA1c only was returning an adjusted R-squared of 0.41 and addition of DBP increased R-squared to 0.56:

$$elnGDR = 3.091 - 0.141 * HbA1c \quad (40)$$

$$elnGDR = 3.842 - 0.125 * HbA1c - 0.012 * DBP \quad (41)$$

The authors have not yet cross-validated this model. The manuscript has been cited nine times to date (Nov 2021). The formula was used in the assessment of metabolic control in people with earlier onset of type 1 diabetes, showing an increased insulin resistance (Wei et al. 2020), and in the characterization of people with fulminant type 1 diabetes (Yang et al. 2020b).

Also in 2018, *Stawiski et al.* presented a calculator for insulin resistance estimation based on neural network analysis of the results of 315 clamps performed in people aged 7.6 to 19.7 years with type 1 diabetes (Stawiski et al. 2018). Validation of their model using testing set of data (training: testing split of data 20:80 ratio) showed R-squared correlation with clamp-derived GDR of 0.66. Variables chosen by the artificial neural network procedure were BP (categorized as normal, elevated, or hypertension), Tanner stage of puberty (categorized 1 to 5), HDL-C, sex, BMI (SDS), TDD, and HbA1c. As this procedure was based on

neural network algorithm, there was no "formula" presented, but authors created a calculator based on this modelling. Unfortunately, it seems no longer to be available (Nov 2021).

Our group (Januszewski et al. 2020) published a set of formulae estimating GDR, M/I, and log M/I using clinical, demographic, and laboratory parameters (Januszewski et al. 2020, 2021). We derived them from 28 people with type 1 diabetes. The GDR range measured during the last 60 min of clamp was 1.9 to 14.1 mg/kg/min. We established that GDR, M/I, and $\log_{10}$M/I measured in the last 30, 60, and 90 min of the clamp correlated well with each other with correlation coefficients (Pearson) varying between 0.64 and 0.99 (all $p < 0.0001$) (Januszewski et al. 2020). We also measured 38 biomarkers, including novel risk factors related to inflammation and adipokines, and applied an exhaustive search procedure to derive the best model (based on adjusted R-squared criterion) estimating GDR, M/I, and $\log_{10}$M/I.

Our best formulae for each of those parameters were:

$$eGDR = -31.714 + 0.142 * Age + 2.399 * \text{HDL} - \text{C} + 8.613 * 1neGFR - 2.051 * VAI - 0.12 * PP \quad (42)$$

Adjusted R-squared 0.46

$$elnGDR = 0.861 + 0.705 * Sex + 0.012 * Age - 0.101 * HbA1c + 1.204 * \text{HDL} - \text{C} - 0.017 * PP \quad (43)$$

Adjusted R-squared 0.40

$$eM/I = 0.052 - 0.939 * 10^{-4} * Sel + 0.506 * 10^{-3} * T1D\ dur. * 0.269 * 10^{-4} * Lens\ FL - 0.146 * 10^{-2} * BMI \quad (44)$$

Adjusted R-squared 0.51

$$eM/I = 0.015 + 0.103 * 10^{-3} * Adiponectin - 0.378 * 10^{-3} * Leptin + 0.229 * 10^{-2} * RAGE - 0.062 * ICAM \quad (45)$$

Adjusted R-squared 0.51

$$elog_{10}M/I = -1.316 + 0.295 * Sex + 0.428 * \text{HDL} - \text{C} - 0.011 * PP + 0.008 * T1D\ dur. - 0.019 * WH \quad (46)$$

Adjusted R-squared 0.66

$$e\ log_{10}M/I = -2.266 - 0.047 * CRP + 0.314 * 10^{-2} * Adiponectin - 1.285 * 10^{-2} * Leptin + 0.08 * \text{IL} - 6 \quad (47)$$

Adjusted R-squared 0.62

$$e\,log_{10}M/I = -2.119 - 0.243 * TG + 0.322 * 10^{-2} * Adiponectin - 0.943 * 10^{-2} * Leptin + 0.077 * \mathrm{IL} - 6 \quad (48)$$

Adjusted R-squared 0.62

$$e\,log_{10}M/I = -1.299 + 0.295 * Sex + 0.546 * \mathrm{HDL} - \mathrm{C} - 0.029 * BMI - 0.057 * HbA1c - 0.503 * WHR \quad (49)$$

Adjusted R-squared 0.60

In the formulae above, the measurements, abbreviations, and units used were age (yrs.); HDL-C (mM); eGFR (calculated from Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (Levey et al. 2009)); VAI, visceral adiposity index, a gender-specific index, based on BMI, waist circumference, triglycerides, and HDL-C and which forms an indication of fat distribution and function (Amato et al. 2010); PP, pulse pressure (mmHg); sex (men = 1, woman = 0); HbA1c (%); Sel, soluble eSelectin; CD62E (ng/mL); T1D dur, type 1 diabetes duration (yrs.); Lens FL, eye lens crystalline autofluorescence (AU) (Januszewski et al. 2012); BMI, body mass index (kg/m^2); adiponectin; Acrp30 (mg/mL); leptin (μg/mL); RAGE, receptor for advanced glycation end products (ng/mL); ICAM; CD54, soluble intercellular cell adhesion molecule 1 (μg/mL); WH, waist-to-height ratio, a measure of the distribution of body fat (Lee et al. 2008); CRP, high-sensitivity C-reactive protein (mg/L); TG, triglycerides (mM); and WHR, waist-to-hip ratio. The manuscript has been cited two times to date (Nov 2021).

We initially validated our formulae statistically using internal fivefold cross-validation (repeated 100 times) and Harrell's bootstrap-based (1000 bootstraps) optimism-corrected calculation of R-squared (Harrell et al. 1996). The former resulted in R-squared values being similar to adjusted R-square calculated from "full" model, and optimism-corrected R-squared was, not surprisingly, on average 33% lower (R-squared varying between 33% and 95% values of "full" model R-squared).

Further to that, in 2021, we have performed validation of our models and also Williams' (Williams et al. 2000), Zheng's (Zheng et al. 2017), and Dabelea's (Dabelea et al. 2011) models, using additional dataset from external source providing data from clamp studies of 104 adults with type 1 diabetes (Januszewski et al. 2021). The parameters of interest were measured during the last 30 min. of the clamp. The elog$_{10}$M/I calculated from the two models correlated with GDR measured during the clamp with Deming R-squared of 0.31 and 0.40, respectively. In the same comparison of other GDR formulae, the Deming R-squared values (eGDR calculated from the formula vs. GDR measured during the clamp) were between 0.22 and 0.24.

All the formulae presented herein provide an approximation of GDR obtained during euglycemic-hyperinsulinemic clamps. It is however apparent that none of them can be used to classify a person as having low or high GDR. While useful in

clinical research, the approximation of GDR with an adjusted R-squared approx. 0.60 is not accurate enough to form the basis of a clinical decision, such as the use of adjunct insulin sensitizing therapy, such as metformin, in people with type 1 diabetes (Livingstone et al. 2017). Nevertheless, all those formulae have shown reduced GDR in people with type 1 diabetes with versus without microvascular complications, in a similar way as the GDR measured in the clamp. Only McAuley et al. attempted to transform their formula into the score (logistic regression), which can be used to calculate probability of insulin resistance (determined as Mffm/I $\leq$ 6.3 M/mU/L) (Mcauley et al. 2001). Although their internal validation (bootstrap) showed marginal deterioration in sensitivity and specificity, it was much more prominent if Mffm/I was determined as a categorical variable. This is a common problem when formula(e) is/are derived from relatively small sample sets, which is common given the cost, labor, and expertise intensive nature of clamp studies. The approximations are not as accurate to divide individuals into two groups on a new sample set (validation set) as on the sample used to establish the score (training set).

We have assessed the ability of all five formulae presented here to discriminate between people with type 1 diabetes with clamp measured GDR <4 mg/kg/min and above and < 5.6 mg/kg/min and above. AUC (J statistics) for the former varied between 0.55 (Williams et al. 2000) and 0.64 (Januszewski et al. 2021) (elnGDR formula 43), and for the latter, the range was between 0.54 (Williams et al. 2000) and 0.76 (Januszewski et al. 2021) (elog$_{10}$M/I formula 49).

Application to Prognosis and Other Diseases or Conditions

Higher insulin resistance is associated with increased risk of pre-diabetes, gestational diabetes, and type 2 diabetes and can also coexist with type 1 diabetes. Insulin resistance varies widely between people and even within an individual over time. Genetic factors; body habitus; lipid levels, particularly triglyceride and HDL levels; intercurrent illness; medications; hormonal factors; pregnancy; growth spurts and other hormones, such as insulin resistance-inducing glucagon and cortisol; physical activity; and psychological stress impact insulin resistance. Other conditions associated with insulin resistance include acanthosis nigricans, polycystic ovary syndrome, and some drugs, such as steroids and chemotherapeutics used in cancer treatments.

Insulin resistance is often associated with adverse short- and long-term outcomes, including larger body habitus, need for higher insulin doses, and higher risk of chronic complications, including microvascular (retinopathy (eye), kidney, and nerve damage) and macrovascular complications (heart disease, stroke, and amputations), and of death. In type 1 diabetes, the delivery of pharmacologic doses of insulin to the subcutaneous tissues induces insulin resistance, rather than the natural endogenous delivery of lower doses of insulin.

With the diabetes pandemic and increasing prevalence of people with type 1 diabetes, an estimated one in ten adults in the world today has diabetes (International Diabetes Federation 2021). The personal and socioeconomic costs rise with complications, hence with insulin resistance. In this modern, and still evolving, era of precision medicine, the means to measure, monitor, and treat (if high) insulin resistance are desirable. Insulin clamp studies remain the gold standard, but are clearly a research tool for those with the available expertise and resources. Modern versions of clamp studies include isotopes, enabling delineation of hepatic and muscle insulin resistance. Means to estimate insulin resistance reliably are still needed for larger clinical research studies and for clinical practice. Equations, such as those presented herein (Table 1), and future even more accurate measures that are well-validated against gold standards and in various studies in different groups are desirable.

Weight loss, if needed, increased physical activity, insulin delivery means, treatment of hypertriglyceridemia, intercurrent illnesses, and adjunct therapy, currently mainly metformin and thiazolidinediones, in people with type 1 diabetes may be useful means.

At this stage, we know that insulin resistance is associated with an adverse prognosis, and we know much about some driving factors. What we need to know more are practical ways to measure it, especially in the clinic, and even more importantly the best means to prevent or treat it. We hope this chapter provides some guidance and stimulates thought and research.

Mini-Dictionary of Terms

Euglycemic-hyperinsulinemic clamp – a research procedure in which blood insulin concentration is increased to supraphysiological levels with euglycemia maintained through intravenous glucose infusion at a variable rate. The glucose infusion rate is indicative of whole-body insulin action. Enhanced insulin action (i.e., greater insulin sensitivity) requires a greater glucose infusion rate. It is important to note that the manner in which an insulin clamp is performed can significantly affect the results obtained.

OGTT – oral glucose tolerance test. Currently the gold standard for the diagnosis of diabetes or of pre-diabetes. Interpretation is based on venous plasma glucose results obtained before and 2 h after a 75 g oral glucose load. Different glucose loads are used in some countries, and diagnostic cut-points are usually lower in pregnancy.

Adjusted R-squared – a modified version of "regular" R-squared that has been adjusted for the number of predictors in the model. Specifically, adjusted R-squared is equal to 1 - (n - 1)/(n – k - 1) *(1-R-squared), where n is the sample size and k is the number of independent variables. Adding more independent variables or predictors to a regression model tends to increase the R-squared value, which tempts makers of the model to add even more variables. This is called overfitting and can return an unwarranted high R-squared value. Adjusted R-squared is used to determine how

Table 1 Variables used to estimate insulin sensitivity/insulin resistance/glucose disposal rate

Number of equation	Age	Sex	Diabetes duration	Blood pressure	Insulin/ TDD	Glucose	HbA1/ HbA1c	TG	HDL-C	Body habitus	Research biomarkers	eGFR
3					X	X						
4					X	X						
5					X	X				X		
6					X	X				X		
7					X	X						
8					X	X						
9					X	X						
10					X	X						
11					X	X						
12					X	X						
13	X				X					X		
14					X	X						
15					X	X				X		
16					X			X		X		
17					X			X				
18					X			X		X		
19					X			X				
20					X	X						
21								X	X			
22								X	X	X		
24				X						X		
25				X			X			X		
26				X			X			X		
27				X			X			X		

(continued)

Table 1 (continued)

Number of equation	Age	Sex	Diabetes duration	Blood pressure	Insulin/ TDD	Glucose	HbA1/ HbA1c	TG	HDL-C	Body habitus	Research biomarkers	eGFR
28				X			X			X		
29							X	X		X		
30										X		
31				X	X			X		X	X	
33				X	X			X		X		
35				X		X	X	X		X	X	
37		X				X	X	X		X		
39				X			X			X		
40							X					
41				X			X					
42	X			X					X	X		X
43	X	X		X			X		X			
44			X							X	X	
45											X	
46		X	X	X					X	X		
47											X	
48								X			X	
49		X					X		X	X		

Blood pressure: SBP, DBP as continuous variables or presence of hypertension
Body habitus: weight, WC, WHR, WH, VAI, BMI
Research biomarkers: adiponectin, selectin, high-sensitivity CRP, RAGE, lens crystalline fluorescence, ICAM-1, leptin, IL-6

reliable the correlation is and how much it is determined by the addition of independent variables (Karch 2020).

Bootstrapped optimism-corrected prediction – a method for an estimation of "optimism" involved in the C-statistic measurement due to overfitting of the model. It requires building up a model ("original") using all available data and calculating model's performance. Then multiple subsets of samples are being created using bootstrap with replacement (containing predicting variables and response variable). The model is built again, using the same procedure, in each of the subset datasets and each "new" model's performance is calculated. Each of the "new" models is then applied to the full dataset and model performance is calculated. Optimism is defined as a difference between performance of the "original" model on the subset of data and performance of the "new" model on the subset of data. The average of these values is the optimism of the "original" model. The optimism-corrected performance of the "original" model is then reduced by this average. This result value is a nearly unbiased estimate of the expected values of the optimism that would be obtained in external validation (Harrell et al. 1996; Smith et al. 2014; Iba et al. 2021).

Deming regression – a technique for comparing two measurements (done using, e.g., two different methods) by fitting a straight line to two-dimensional data where both variables, X and Y, are measured with error. This is different from simple linear regression where only the response variable, Y, is measured with error. Deming regression is often used for method comparison studies in clinical chemistry to look for systematic differences between two measurement methods (Deming 1943).

Stepwise linear regression – a method of fitting regression models in which the choice of predictive variables is carried out by an automatic procedure. In each step, a variable is considered for addition to or subtraction from the set of explanatory variables based on some prespecified criterion (Efroymson 1960). A fundamental problem with stepwise regression is that some real explanatory variables that have causal effects on the dependent variable may happen to not be statistically significant, while nuisance variables may be coincidentally significant. As a result, the model may fit the data well in-sample, but do poorly out-of-sample (Smith 2018).

Sensitivity – the ability of a test to correctly identify person with a condition/disease.

Specificity – the ability of a test to correctly identify person without a condition/disease.

Cross-validation – a model validation technique aiming at assessing how the statistical model or the results of analysis will perform in an independent dataset.

Exhaustive search – an algorithm which examines every combination of variables of interest to find the best solution based on specified metrics (e.g., adjusted R-squared). The main strength is that it is guaranteed to find the best model (from specified variables). The main disadvantage is that it is computationally demanding (e.g., if one wants to get the best model containing 5 variables chosen from a dataset containing 40 variables, the procedure must calculate results of 658,008 combinations).

Key Facts

- Insulin sensitivity is most accurately measured by euglycemic-hyperinsulinemic clamp studies but can also be estimated using mathematical formulae utilizing various biomarkers.
- Variables most often used in the calculation of insulin sensitivity are those reflecting body habitus, blood pressure, glycemia, and lipids.
- Depending on the number and type of variables used, the formulae to calculate insulin sensitivity estimates are explaining approximately 60% of the insulin sensitivity variance observed in the clamp study data from which were used to calculate these formulae.
- In independent validation studies, the insulin sensitivity estimation formulae showed lower agreement with clamp data of 20–50%.

Summary Points

- In this chapter, we have presented current approaches toward the assessment of insulin sensitivity/insulin resistance/glucose disposal rate, including in people with type 1 diabetes.
- Those measurements are done either by creating specific "new" indexes or by approximation (using various statistical techniques) of the parameters calculated from a euglycemic-hyperinsulinemic clamp study, which remains the "gold standard" for the assessment of glucose utilization.
- It must be emphasized that the latter approach is often lacking independent validation. Although the values calculated from these formulae are showing associations with various diabetes-related pathologies, it must be noted that specific IS/IR/eGDR thresholds for the parameters calculated from these formulae have never been defined and validated.
- Given the association between insulin resistance and the development of type 2 diabetes, the chronic complications of type 1 diabetes and the availability of adjunct therapies which may mitigate insulin resistance further studies are merited.

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Serum Betatrophin: What It Shows and How It Alters in Gestational Diabetes Mellitus

17

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Contents

Gestational Diabetes Mellitus: Definition and Prevalence 377
GDM Incidence 378
GDM Risk Factors 378
GDM Treatment 378
Glucose Regulation During Healthy Pregnancy 379
GDM Physiology 379
The Adipose Tissue in the Pathophysiology of Gestational Diabetes Mellitus 380
Betatrophin 381
Betatrophin in Gestational Diabetes Mellitus 385
Applications to Prognosis, Other Diseases or Conditions 387
Applications to Prognosis 388
Applications to Other Diseases or Conditions 389
Mini Dictionary of Terms 390
Key Facts of Betatrophin 391
Summary Points 391
References 391

Abstract

Gestational diabetes mellitus is defined as diabetes mellitus diagnosed during the second or third trimester of pregnancy and not clearly associated with underlying type 1 or type 2 diabetes. According to the most recent data from the International Diabetes Federation, gestational diabetes mellitus affects 16.7% of all pregnant women, and approximately 21.1 million babies are born to pregnant women with

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diabetes each year. There are many factors that contribute to the development of gestational diabetes, but the most common is beta-cell dysfunction and increased insulin resistance during pregnancy. In both animal and human studies, betatrophin has been found to be a major factor in beta cell growth and enlargement and has been associated with an increased risk of developing type 2 diabetes. While the effect of betatrophin on beta-cell proliferation in humans is still unknown, there has been growing interest in determining circulating levels of betatrophin in patients to elucidate its putative function in metabolic diseases. Several studies have shown that betatrophin concentrations are increased in both maternal serum and cord blood in pregnant women with gestational diabetes mellitus. Although there are some differences between studies, most likely due to the small sample sizes and wide range of detection rates for betatrophin levels, betatrophin is a promising diagnostic marker for gestational diabetes mellitus. Further research may help us to better understand the role of betatrophin in the pathophysiology of gestational diabetes mellitus.

Keywords

Adipokine · Angiopoietin-like protein 8 · Batokine · Betatrophin · Gestational diabetes · Insulin resistance · Lipasin · Obesity · Placental lactogen · Pregnancy · Refeeding-induced fat and liver

List of Abbreviations

ADA	American Diabetes Association
AKT	Protein Kinase B
AMPK	AMP-activated protein kinase
ANGPTL8	Angiopoietin-like protein 8
ASP	Acylation-stimulating protein
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BMP	Bone Morphogenetic Proteins
BMP4	Bone morphogenetic protein 4
CXCL14	Chemokine Ligand 14
ELISA	Enzyme-linked immunosorbent assay
FABP-4	Fatty acid-binding protein 4
FGF21	Fibroblast Growth Factor 21
GDM	Gestational Diabetes Mellitus
GLUT4	Glucose Transporter 4
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
HOMA-IR	Homeostasis model assessment of insulin resistance value
IADPSG	International Association of Diabetes and Pregnancy Study Group
IGF-1	Insulin Growth Factor-1
IGFBP-2	Insulin-like growth factor-binding protein 2

IL	Interleukin
IRS-1	Insulin Receptor Substrate 1
LDL	Low-density lipoprotein
LPL	Lipoproetin Lipase
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
METRNL	Meteorin-Like
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NRG4	Neuregulin-4
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PPAR-alpha	Peroxisome proliferator-activated receptor
RBP4	Retinol-binding protein 4
RIFL	Refeeding-induced fat and liver
SFRP5	Secreted Frizzled-Related Protein 5
T3	Triiodothyronine
TNF	Tumor necrosis factor
UCP1	Uncoupling protein
VEGF-A	Vascular Endothelial Growth Factor A

Gestational Diabetes Mellitus: Definition and Prevalence

The American Diabetes Association (ADA) formally defines gestational diabetes mellitus (GDM) as "diabetes mellitus diagnosed during the second or third trimester of pregnancy that is not definitely associated with underlying type 1 or type 2 diabetes" (American Diabetes Association 2018). However, the precise cutoff serum glucose levels for diagnosing GDM vary depending on the criteria used, and there has thus far been a lack of consistency among medical practitioners. The International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria are currently recommended by the ADA, the World Health Organization, the International Federation of Gynecology and Obstetrics, and the Endocrine Society for the diagnosis of GDM (Chiefari et al. 2017). The IADPSG criteria were developed based on the international multicenter Hyperglycemia and Adverse Pregnancy Outcomes Trial findings, which included 23,000 pregnant women (HAPO Study Cooperative Research Group et al. 2008). The study results revealed a constant risk of unfavorable outcomes for both mother and fetus with uncontrolled maternal glycemia – even when plasma glucose levels were below the diagnostic threshold for GDM – implying that intervention criteria needed to be changed. All pregnant women should have a fasting plasma glucose test at their first prenatal visit (a value of >92 mg/dL is indicative of GDM), and if the fasting plasma glucose does not exceed 92 mg/dL, the IADPSG recommends a 2-h 75 g oral glucose tolerance test (OGTT) at 24–28 weeks gestation. Since these cutoffs are lower than other

previously published guidelines, they have resulted in a significant increase in the number of GDM cases and associated increased medical costs (Egan et al. 2017). Therefore, there has been considerable debate among experts about whether the IADPSG criteria should be modified to only screen at-high risk pregnant women (i.e., women over the age of 35, those with a body mass index (BMI) greater than 25 kg/m^2, those from high-risk ethnicities, or those with a family history of diabetes). Other studies, however, have shown that these efforts may miss a significant proportion of GDM cases while not significantly lowering treatment costs (Capula et al. 2013; Reece and Moore 2013). As a result, the IADPSG criteria are now the most widely recommended guidelines, though alternative criteria are still used in various facilities and countries.

GDM Incidence

Because of the numerous methods used for GDM screening and diagnosis, current estimates of GDM prevalence are difficult to determine. However, according to the most recent (2021) International Diabetes Federation data, GDM affects 16.7% of all pregnant women, resulting in approximately 21.1 million babies are born to pregnant women with diabetes each year (International Diabetes Federation 2021).

GDM Risk Factors

Risk factors for insulin resistance and/or diabetes include maternal age over 40, overweight or obesity, eating a Westernized diet low in micronutrients, and having a family history of diabetes mellitus. Also, several further risk factors for GDM have been consistently identified. Other risk factors that have been found related to GDM are as follows: excessive weight gain in pregnancy period, genetic polymorphism, altered in-utero microenvironment, and other insulin-resistant medical diseases such as polycystic ovarian syndrome (Plows et al. 2018).

GDM Treatment

Lifestyle modification (diet and exercise) is the only widely accepted treatment or prevention method for GDM. Furthermore, due to the common insulin resistance in this population, insulin treatment has only limited utility in the treatment of GDM. Despite the potential for new oral diabetes medications such as glyburide and metformin, there are still concerns about their long-term safety for pregnant women and their children. Therefore, they are now considered as a second-line treatment option for GDM (ACOG Practice Bulletin 2018).

Glucose Regulation During Healthy Pregnancy

At some point in every healthy pregnancy, the pregnant woman's body experiences a series of physiological changes. Additionally, there are modifications to the circulatory and respiratory systems as well as the hematologic and metabolic systems that are affected. Insulin sensitivity is one of the most important metabolic adaptations. Insulin sensitivity changes during pregnancy to accommodate the additional demands of the growing fetus inside. Adipose stores are prepared for the increased energy requirement of pregnancy by improving insulin sensitivity during early gestation. A rise in local and placental hormones, such as estrogen, progesterone, leptin, cortisol, human placental lactogen, and placental growth hormone, led to an increase in insulin resistance that occurs as the pregnancy progresses (Baz et al. 2016).

Therefore, blood glucose levels are slightly raised, and this glucose is easily transferred through the placenta to provide fetal development. In addition, this modest insulin resistance facilitates endogenous glucose synthesis and fat breakdown, resulting in an even more significant rise in maternal serum blood glucose and free fatty acid concentrations. For pregnant women, evidence from animal studies suggests that the pancreas compensates for these alterations through enhanced glucose-stimulated insulin production (Moyce and Dolinsky 2018). The fact that maternal insulin sensitivity returns to pre-pregnancy levels within a few days of delivery highlights the role of placental hormones in this process.

GDM Physiology

Chronic insulin resistance is present in the majority of cases of GDM, which is partially exacerbated by the typical insulin resistance that occurs especially during latter half of pregnancy (Fu and Retnakaran 2022). Consequently, women with this condition have higher levels of insulin resistance than healthy pregnant women, resulting in lower glucose utilization, higher glucose synthesis, and higher serum levels of free fatty acids. Excessive insulin secretion in response to high energy expenditure and high levels of insulin resistance is hypothesized to be the cause of beta-cell degeneration over time and eventually results in exhausting themselves. Given the striking similarity in etiopathology between GDM and type 2 diabetes, there has been substantial disagreement over the two conditions that should be separately considered in terms of etiology (Zajdenverg and Negrato 2017).

There are many factors that contribute to the development of GDM, but the most common is beta-cell dysfunction and increased insulin resistance throughout pregnancy. These impairments typically occur before pregnancy and can progress during pregnancy, and are associated with an increased risk of type 2 diabetes in the post-pregnancy period (Retnakaran et al. 2010).

Insulin resistance occurs when cells are unable to react to insulin stimulation effectively. In most cases, insulin resistance is caused by defects in insulin signaling,

which results in a lack of plasma membrane translocation of glucose transporter 4 (GLUT4) – the primary transporter capable of transporting glucose into the cytoplasm for energy use. In GDM, glucose uptake by insulin stimulation is reduced by 50% when compared to a healthy pregnancy (Kampmann et al. 2019). Even though insulin receptor abundance is frequently unchanged, altered insulin receptor serine/threonine phosphorylation dampens insulin signaling. Furthermore, GDM has been shown to alter the expression or phosphorylation of a number of downstream regulators of insulin signaling, including GLUT-4, insulin receptor substrate 1 (IRS-1), and phosphatidylinositol 3-kinase (PI3K). The majority of these molecular changes continue during pregnancy (Friedman et al. 2008). During a normal pregnancy, beta-cell hyperplasia and hypertrophy occur to meet the metabolic needs of the pregnancy. Beta-cells are unable to adapt to the demands of pregnancy during gestational diabetes, resulting in hyperglycemia when combined with decreased insulin sensitivity. Following pregnancy, beta-cell mass, serum glucose levels, and insulin receptor sensitivity all return to normal levels. Changes in beta-cell mass, insulin receptor sensitivity, and blood glucose levels may recover to normal in the postpartum period or remain impaired, putting them on a path to GDM or type 2 diabetes in subsequent pregnancies.

Furthermore, neurohormonal dysregulation has been linked to the etiology of insulin-resistant diseases like GDM. The neurohormonal system, which regulates hunger, total energy balance, and baseline metabolism, is formed by a complex network of the central nervous system (e.g., cortical regions that regulate intellectual, ocular, and reward centers) and peripheral (e.g., appetite hormones that are secreted from the gastrointestinal tract and adipose tissue) signals (Moehlecke et al. 2016). These factors contribute to GDM by influencing adipose tissue and glucose metabolism. This pathway is heavily influenced by the body's circadian system, which may explain why patients with severe sleep disorders or who work shifts are more likely to develop GDM (Facco et al. 2017). Adipokines and batokines are cell-signaling proteins that are primarily released by adipocytes and regulate neurohormonal cell metabolism.

The Adipose Tissue in the Pathophysiology of Gestational Diabetes Mellitus

Adipose tissue is a fully functional endocrine organ that secretes hundreds of bioactive chemicals known as adipokines. Adipokines have an autocrine and paracrine effect on adipocyte function, allowing adipocytes to interact intensively with the central nervous system, muscle, liver, and pancreas, as well as other organs in a neuroendocrine manner (Oh et al. 2016). Adipose tissue is divided into two depots based on their architecture and functions: brown adipose tissue and white adipose tissue. White adipose tissue is formed by unilocular cells, which have a single lipid droplet and undergo dynamic changes in response to calorie status. Excess calories are deposited in lipid vacuoles as triacylglycerol (lipogenesis), and the deposited triacylglycerol is degraded into glycerol and fatty acids (lipolysis) for use by other

tissues. On the other hand, brown adipose tissue depletes lipids in response to "heat" generated by beta-adrenergic activation or cold exposure. Adipocytes in brown adipose tissue are composed of multilocular cells with smaller lipid droplets and have a high number of mitochondria as well as an increased level of mitochondrial uncoupling protein 1, which is found in the inner membrane of the mitochondria and decouples oxidative respiratory metabolism from adenosine triphosphate (ATP) generation (Saely et al. 2012). White and brown adipose tissues both produce functional peptides and proteins known as "adipokines" and "batokines-also known as brown adipokines," respectively. Because of the structure and activity of these tissues, adipokines derived from white adipose tissue are thought to be biologically distinct from batokines derived from brown adipose tissue. These adipocyte-derived substances function similarly to hormones in that they regulate carbohydrate and lipid metabolism, insulin transmission, and inflammatory processes in various tissues, including the liver, pancreas, muscle, adipose tissue, and brain.

Adipokines are classified as either anti-inflammatory or inflammatory based on their expression levels in obesity and diabetes mellitus. Adiponectin, cardiotrophin-1, omentin-1, and secreted frizzled-related protein 5 (SFRP5) are anti-inflammatory adipokines that improve energy utilization in hepatocytes, muscle cells, pancreatic cells, and adipocytes (Jung and Jung 2021). The classification and function of adipokines are summarized in Table 1.

Batokines are produced by brown adipose tissue as well as brown-like adipose tissue. A wide range of processes, such as thermogenesis, immunity, angiogenesis, substrate consumption, and metabolic health regulation are controlled by batokines. In addition, these batokines conduct autocrine, paracrine, and endocrine actions in distant organs and tissues such as the liver, heart, musculoskeletal, brain, and white adipose tissue. The identification and characterization of factors released by adipose tissues may aid in developing therapeutic candidates for the treatment of a wide range of metabolic diseases, including obesity, diabetes mellitus, atherosclerosis, and dyslipidemia, including gestational diabetes. Table 2 summarizes batokines and their metabolic effects (Lee et al. 2019).

Betatrophin

In both animal and human studies, betatrophin has been found to be an essential factor in the growth and expansion of B-cells, and it has been linked to an increased risk of developing type 2 diabetes. Although it was initially identified as the hepatocellular carcinoma-associated protein TD26, it has also been referred to as Angiopoietin-like protein 8 (ANGPTL8), lipasin, or refeeding-induced fat and liver (RIFL) (Luo and Peng 2018). Because of its ability to stimulate beta-cell proliferation, the term "*betatrophin"* has recently gained popularity. The gene C19orf80 encodes the hormone, which belongs to the angiopoietin-like protein family (it is named as Gm6484 in mice). In humans, it has a more liver-specific expression than in mice, where it is mainly found in the liver and white and brown adipose tissues

Table 1 Classification and role of adipokines

	Adipokines	Metabolic role
Anti-inflammatory adipokines (Lower expression in obesity and diabetes mellitus)	Adiponectin	Gluconeogenesis, steatosis, inflammation, and fibrosis by activating 5′ adenosine monophosphate-activated protein kinase (AMPK) via AdipoR1 receptor in the liver Enhance B-oxidation, glucose uptake, and insulin sensitivity by activating the peroxisome proliferator-activated receptor (PPAR) alpha via AdipoR2 receptor in the liver Enhance B-oxidation, glucose uptake, and insulin sensitivity via AdipoR1 and R2 receptors in the skeletal muscle Increase insulin secretion by activating AMPK and enhance B cell function and proliferation via AdipoR1 receptor in the pancreas
	Omentin-1	Enhance glucose uptake, exercise-induced insulin-sensitizing, and activated insulin receptor substrate by inhibiting the mTOR signaling pathway in the adipocytes
	SFRP5	Increase insulin sensitivity Inhibits obesity, atherosclerosis, and Wnt5a-mediated inflammation via Wnt signaling transduction by binding to Wnt protein
	Cardiotrophin-1	Increase lipolysis and decrease adipocyte size. Improve glucose homeostasis and promote glucose uptake by insulin-stimulated phosphorylation of protein kinase B (AKT) in myotubes and adipocytes
Inflammatory adipokines (Higher expression in obesity and diabetes mellitus)	FABP-4	Increase adiposity and insulin resistance
	ASP	Enhance triglyceride synthesis, reduce fatty acid uptake Increase inflammation and insulin resistance
	RBP4	Increase inflammation and insulin resistance
	Lipocalin-2	Increase inflammation and insulin resistance Enhance adipose tissue remodeling
	Chemerin	Increase adipogenesis and insulin resistance Atherosclerotic vascular changes, endothelial activation, reduced glucose uptake

(continued)

Table 1 (continued)

	Adipokines	Metabolic role
	Visfatin	Enhance inflammation, obesity, adiposity, insulin resistance
	Leptin	Monocyte and macrophage activation Inflammatory stimuli (TNF, IL-6) in monocytes Administration of leptin normalized hyperinsulinemia, hyperglycemia, insulin resistance, lipodystrophy Fatty acid oxidation by activating AMPK in other tissues
	Resistin	Decrease insulin-mediated glucose uptake in adipocytes Enhance hepatic glucose production and hepatic insulin resistance
	Apelin	Inhibition of insulin secretion Deterioration of glucose metabolism Increased size of lipid droplets reduces adipocyte number Administration of apelin improves hepatic fibrosis, cardiac contractility, angiogenesis Promote glucose uptake by activating AMPK and AKT in soleus muscle
	Gremlin-1	Contribute BMP4 resistance Increased in hypertrophic obesity

AKT, protein kinase B; AMPK, AMP-activated protein kinase; ASP, Acylation-stimulating protein; BMP4, Bone morphogenetic protein 4; FABP-4, fatty acid-binding protein 4; IL, Interleukin; mTOR, Mammalian target of rapamycin; PPAR-alpha, Peroxisome proliferator-activated receptor alpha; RBP4, Retinol-binding protein 4; SFRP5, Secreted frizzled-related protein 5, TNF, Tumor necrosis factor

(Ren et al. 2012). Betatrophin expression in mice is affected by both diet (increased synthesis induced by high-fat meal) and heat (enhanced production in cold conditions) (Fu et al. 2013a). Yi et al. administered mice an insulin receptor antagonist (S961) for 1 week to induce carbohydrate intolerance and hyperinsulinemia. Ki67 immunohistochemistry analysis revealed a dose-dependent increase in beta cell proliferation simultaneously. Four days after the S961 therapy was discontinued, beta-cell mass growth rates returned to normal. When S961 was incubated, it had no effect on the proliferation of isolated mouse islets. According to the findings of a microarray study of those tissues, betatrophin (Gm6484) was found to be expressed at higher levels in the mice's liver and white adipose tissue after S961 treatment. Betatrophin messenger ribonucleic acid (mRNA) expression was also found to be higher in *ob/ob* and *db/db* mice, as well as pregnant mice. On the other hand, betatrophin levels did not increase in mice with acute beta-cell depletion caused by beta cell-specific diphtheria toxin exposure. They also showed that betatrophin hormone is secreted and found in human plasma. Overexpression of betatrophin in the liver of mice resulted in a 17-fold increase in beta-cell proliferation and a 3-fold

Table 2 Summary of batokines and their metabolic effects

Batokines	Metabolic role
FGF-21	Cardioprotective effects regulate glucose and lipid metabolism and energy homeostasis, promotes thermogenesis
T3	Contributes to many physiological processes, including metabolism, thermogenesis, development, and heart rate
IGFBP-2	Involves in cellular processes, such as proliferation and migration, and links the communication between energy metabolism and bone formation
IGF-1	Mitigates pro-inflammation and ameliorates glucose homeostasis
CXCL-14	Enables immune cells (monocytes, dendritic cells, natural killer cells) to localize at inflammatory sites in response to inflammation
VEGF-A	Promotes vascularization, enhances thermogenesis
NRG-4	Represses hepatic lipogenesis, protects hepatic steatosis and hyperlipidemia Inversely associated with nonalcoholic fatty liver disease, type 2 diabetes, GDM, and metabolic syndrome
Myostatin	Negative effects on muscles and brown adipogenesis. Induction of myostatin causes impaired insulin-stimulated glucose uptake and insulin resistance
METRNL	Stimulates the recruitment of eosinophils and alternatively activated macrophages in white adipose tissue, and thus increases the level of catecholamines
BMP-8b	Promotes brown adipocyte differentiation Regulates thermogenic processes via signaling through the hypothalamus of the brain
IL-6	Brown adipose tissue-derived IL-6 is essential for the profound effects of brown adipose tissue transplantation on insulin sensitivity and glucose homeostasis.
Endothelin 1	Negatively regulates brown/beige adipogenesis, suppresses UCP1 expression and whole-body energy expenditure
SLIT2-C	Stimulates adipose thermogenesis, enhances energy expenditure, and improves glucose homeostasis
Betatrophin	Represses the activity of lipoprotein lipase, enhances pancreatic βcell replication

BMP, Bone Morphogenetic Proteins; CXCL14, Chemokine Ligand 14; FGF21, fibroblast growth factor 21; GDM, Gestational diabetes mellitus; IGF-1, Insulin Growth Factor-1; IGFBP-2, Insulin-like growth factor-binding protein 2; IL, Interleukin; METRNL, Meteorin-Like; NRG4, Neuregulin-4; T3, Triiodothyronine; UCP1, uncoupling protein; VEGF-A, Vascular Endothelial Growth Factor A

increase in beta-cell mass. Overexpression of betatrophin also resulted in lower fasting blood glucose, better glycemic control, and a normal insulin tolerance response (Yi et al. 2013).

The effect of betatrophin on human beta-cell proliferation is still unknown. Human and mouse islets were implanted under the renal capsules of mice and treated with the insulin receptor antagonist S961 to induce insulin resistance, which has previously been shown to enhance betatrophin production in hepatic tissue. Both native and transplanted mouse islets showed increased beta-cell proliferation, whereas human beta-cells were unaffected. When analyzing the age dependence of human beta-cell growth, it is worth noting that one of the five donors was only

4 years old, another was only 18, and the rest were over 40 (Espes et al. 2015). In conclusion, the involvement of betatrophin in human and mouse beta-cell proliferation has been contested; nonetheless, its role in insulin resistance has become well established, despite the need for additional research into its mode of action.

Concurrently, another study group revealed that betatrophin might inhibit lipoprotein lipase (LPL) activity in vitro. In mice, overexpression of betatrophin resulted in a fivefold increase in blood triglyceride levels. Betatrophin expression in the liver was also increased by a high-fat diet and decreased by fasting. They also demonstrated that cold modulates its expression by promoting its production in brown adipose tissue after cold exposure. According to previous studies, betatrophin is predominantly located in the liver, white and brown adipose tissues. High fat consumption and cold exposure can both increase the level of betatrophin in the body. Furthermore, betatrophin modulates plasma triglyceride levels by suppressing LPL activity, which is accomplished through its interaction with the N-terminal domain of ANGPTL3 (Fu et al. 2013b).

Betatrophin in Gestational Diabetes Mellitus

Betatrophin has been shown to be elevated in pregnant women with GDM as well as in adults with type 2 diabetes in both maternal serum and cord blood, as summarized in Table 3. Firstly, in a mouse model, betatrophin was found to be elevated during gestation. According to the findings of Yi and colleagues, mice at 18.5 date post-conception had roughly 20 times more betatrophin levels than those that were not pregnant or in their early stages of pregnancy (Yi et al. 2013). A physiological compensatory mechanism to induce beta-cell proliferation has been proposed to cause elevated betatrophin levels during pregnancy.

During pregnancy, maternal hormones vary, resulting in a decrease in insulin sensitivity comparable to that found in type 2 diabetes. Because of sufficient cell compensation, most pregnant women's serum glucose levels remain steady during pregnancy. If insulin resistance exceeds what the beta-cells can tolerate or beta-cell function declines, there is a risk of developing GDM. Increased betatrophin levels have been proposed as a way to compensate for the increased insulin requirement. In line with this idea, Ebert et al. observed that betatrophin levels were considerably more significant in women with GDM compared to healthy pregnant women (Ebert et al. 2015). They also found a lower postpartum level of betatrophin than during pregnancy. Pregnant women with GDM reported significantly higher betatrophin levels than their healthy counterparts, regardless of BMI or gestational age (Erol et al. 2015). Previous research found that women with GDM had higher levels of betatrophin than women who did not have diabetes (Wawrusiewicz-Kurylonek et al. 2015a; Xie et al. 2016). Betatrophin has been associated with insulin resistance in patients with type 2 diabetes mellitus, and it has been postulated that it could be a potential novel biomarker for carbohydrate and lipid metabolism.

As a result, proposed markers like betatrophin for detecting the onset of diabetes would help us better understand the disease's underlying pathophysiology and,

Table 3 List of studies that investigated betatrophin levels in pregnant women with GDM

Study	Country	Sample size	Sample	Method/Kit	Trimester	Result
Abdeltawab et al. (2021)	Egypt	109	Serum	Both real-time-PCR and Elabscience ELISA kit	Second/ third	Higher betatrophin level in the GDM women
Ebert et al. (2015)	Germany	148	Serum	Phoenix Pharmaceuticals ELISA kit.	Third	Higher betatrophin levels in women with GDM
Erol et al. (2015)	Turkey	90	Serum	EIAAB ELISA kit	Second	Higher betatrophin level in the GDM women
Seyhanli et al. (2021)	Turkey	90	Serum	CUSABIO ELISA kit	Second	Higher betatrophin levels in women with GDM
Gülcü Bulmuş et al. (2020)	Turkey	60	Serum	Elabscience ELISA kit	Second	Higher betatrophin levels in pregnant women with GDM
Huang et al. (2016)	China	49	Serum	EIAab Science ELISA kit	Second	Higher betatrophin level in the GDM women
Pan et al. (2019)	China	400	Serum	EIAab Science ELISA kit	Second	Higher betatrophin level in the GDM women
Wawrusiewicz-Kuryloneket et al. (2015b)	Poland	190	Serum/ cord blood	USCN Life Science ELISA kit	Third	Higher maternal and cord blood betatrophin levels in women with GDM
Trebotic et al. (2015)	Austria	40	Plasma	EIAAB ELISA kit.	Third	Higher betatrophin level in the GDM women
Xie et al. (2016)	China	54	Cord blood	EIAAB ELISA kit.	Third	Higher betatrophin level in cord blood of women with GDM
Yang et al. (2021)	China	77	Serum/ cord blood	USCNK ELISA kit	Third	Lower maternal and cord blood betatrophin levels in women with GDM

ELISA, Enzyme-linked immunosorbent assay, GDM, Gestational diabetes mellitus, PCR, Polymerase chain reaction

potentially, give solutions for disease prevention. Betatrophin is a promising diagnostic marker for both type 2 diabetes and GDM. The majority of research indicates that betatrophin levels are higher in patients with type 2 diabetes, type 1 diabetes, and GDM. Increased serum levels of betatrophin have also been seen in patients who are obese or have metabolic syndrome. However, certain differences exist between studies, which are most likely due to the small sample sizes and the wide range of detection kits used to test for betatrophin levels. In addition, because the majority of current research is cross-sectional, it is unknown what role betatrophin plays in the pathophysiology of diabetes and how it contributes to beta-cell proliferation and insulin resistance. Further betatrophin researches could help us better understand the biology of diabetes mellitus and other metabolic diseases, leading to new treatment options (Abu-Farha et al. 2017).

Applications to Prognosis, Other Diseases or Conditions

In recent years, there has been a rise in interest in assessing betatrophin circulating concentrations in patients as part of efforts to elucidate its putative function in metabolic disease (Fenzl et al. 2014). Betatrophin levels have been observed to be altered in a number of physiological (e.g., postprandial state) and pathological circumstances such as type 2 diabetes, type 1 diabetes, and obesity (Fu et al. 2014a). There was also a strong relationship between betatrophin levels and metabolic parameters such as BMI, blood glucose levels, insulin resistance, and serum lipid parameters like low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol, and triglyceride levels. However, the findings of these researches show a wide range of variations. Circulating betatrophin levels, for example, ranged from 0.3 ng/ml to 45 ng/ml in lean and nondiabetic patients, and levels were either raised or decreased in type 2 diabetes or obesity. Betatrophin levels were also either positively or negatively associated with insulin levels and correlated with either atherogenic lipid parameters or HDL cholesterol (Gómez-Ambrosi et al. 2014). A recent study of 30 human subjects found that associations between betatrophin and BMI can be both positive and negative, depending on whether antibodies are employed to detect the C-terminal or N-terminal betatrophin. It has been argued that evaluating separate betatrophin species using enzyme-linked immunosorbent assay (ELISA) kits that use either N- or C-terminal antibodies can resolve these contradictions (Fu et al. 2014b). The C-terminal antibody detected both full-length protein (band position corresponding to 22kD) and C terminal fragments (band position corresponding to low molecular weight) when human sera were tested by Western blot. By high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), the smaller pieces were identified as amino acids 118–198 and 133–198, respectively, indicating that betatrophin is likely cleaved in vivo to release the C-terminal fragments while the N-terminal fragments are eliminated (Chang et al. 2013). As a result, the N-terminal test is expected to detect the full-length protein, but the C-terminal kit analyzes the whole betatrophin molecule, which includes both the full-length protein

and the C-terminal fragments. Furthermore, betatrophin levels indicated by the C-terminal kit in lean and nondiabetic subjects are higher than those reported by the N-terminal kit.

Two possible reasons have been proposed for this variation. Splicing of betatrophin transcripts is the first alternative reason. Despite the fact that the Ensemble database contains two other transcripts in addition to the 198-residue transcript ENSP00000252453: isoforms ENSP00000464941 and ENSP00000465378 (lacking 99 and 140 N-terminal amino acid residues, respectively), only the complete transcript was found in human liver (Zhang and Abou-Samra 2014), ruling out alternative splicing. Inconsistencies could also be the result of sample deterioration. Because C-terminal fragments are more stable than N-terminal fragments, they concentrate in the circulation, resulting in elevated serum total betatrophin levels. However, proteolytic regulation appears to be the most likely explanation, given the presence of proteinase cleavage sites and the related C-terminal fragments detected in vivo using mass spectrometry (Chang et al. 2013). Whichever explanation is adopted, care should be taken to examine which antibodies were employed when analyzing betatrophin ELISA data.

Numerous studies have been conducted on betatrophin, and its correlates in nondiabetic individuals. The most extensive study on betatrophin levels in nondiabetic patients found a positive correlation between betatrophin and age, BMI, waist/hip ratio, fasting plasma glucose, glycosylated hemoglobin, serum insulin levels, homeostasis model assessment of insulin resistance value (HOMA-IR), and serum triglyceride levels (Espes et al. 2015).

Previous research has revealed that plasma betatrophin levels increase with age, although there is no association with fasting hyperglycemia, hemoglobin A1c, BMI, or serum lipid profile. In nondiabetic people, betatrophin and age have been found to have a positive correlation. In children over the age of 8, betatrophin levels were shown to be higher in boys than in girls with the same BMI. In individuals without diabetes, some studies reported a negative link between betatrophin levels and BMI, whereas others found a positive correlation. Consistent with recent findings on betatrophin mRNA expression in rodents, circulatory betatrophin levels in lean human individuals were found to increase 2 h after a specific meal (Espes et al. 2015; Fu et al. 2014a).

Applications to Prognosis

The betatrophin mRNA levels in the liver of the *ob/ob* and *db/db* mouse models of obesity and type 2 diabetes were found elevated in the initial study conducted by Melton et al. (Yi et al. 2013). And they found that the expression of betatrophin increased as a response to insulin resistance produced by treatment with the insulin receptor antagonist S961. A further benefit of betatrophin overexpression is an improvement in glycemic management. These data suggest that betatrophin itself does not cause insulin resistance but instead leads to increase beta-cell mass to keep glucose homeostasis normal. Therefore, an increase in betatrophin would be

expected in insulin-resistant patients to prevent the development of clinical diabetes if betatrophin plays the same or a different role in humans (Yi et al. 2013). According to a study by Chen et al., patients recently diagnosed with type 2 diabetes had higher betatrophin levels than healthy controls, patients with impaired fasting blood glucose, and patients with impaired glucose tolerance. Although postmortem studies show a reduction in beta-cell mass in patients with long-term type 2 diabetes, betatrophin is consistently associated with the duration of diabetes (Chen et al. 2015). Researchers have found no difference in glycemic control between people with type 2 diabetes who have higher or lower betatrophin levels in cross-sectional studies, and some have even found that betatrophin levels are higher in those who have poorer glycemic control (e.g., higher fasting blood glucose, HbA1c) (Fu et al. 2014a).

However, the results of these studies are influenced by the different treatment regimens used in the various research populations, and the effects of the most commonly used antidiabetic drugs on betatrophin levels remain to be determined. Two studies found a positive association between betatrophin and HOMA-IR, whereas others found a negative association. Two of these studies focused on newly diagnosed type 2 diabetes individuals, whereas the other study examined patients with newly diagnosed but early-onset type 2 diabetes. Correlations were calculated for all participants in these studies. In one study, there was no change in betatrophin levels between individuals with normal glucose tolerance and those with impaired glucose tolerance. The possible reasons for these contradictory results have been poorly described (Kong et al. 2017).

Several investigations have indicated that betatrophin levels are higher in women with GDM than in control participants, suggesting that increased insulin resistance and insulin requirements in GDM may play a role in the increase in betatrophin levels. Additionally, Natalia et al. and Wang et al. found that betatrophin levels in both maternal serum and umbilical cord blood were elevated in GDM patients. In addition, the quantity of betatrophin in cord blood proved to be higher than in maternal serum, which may indicate its role in increasing beta-cell proliferation during intrauterine life in these studies. Patients with GDM had higher levels of betatrophin in their blood, according to the results of the most recent meta-analysis (Guo and Wang 2016; Wawrusiewicz-Kurylonek et al. 2015b). Betatrophin could be a valuable tool in diagnosing GDM because of the lack of evident symptoms in women with GDM. This finding could help physicians educate GDM patients on preventing disease progression. However, in humans, the regulation and metabolism of betatrophin remain largely unexplored.

Applications to Other Diseases or Conditions

In the insulin resistance pathway, both the liver and adipose tissue produce betatrophin. The liver is a major player in the body's glucose metabolism. Another essential role that adipose tissue plays in insulin resistance is that it causes an increase in inflammation, produces free fatty acids, and inhibits the formation of

adiponectin. Since obesity is associated with insulin resistance and abnormal fat metabolism, it is critical that betatrophin is also controlled by adipose tissue (O'Rahilly 2007). Studies in rodents have shown that obese rats have increased betatrophin levels. In nondiabetic subjects, a recent study found a positive correlation between betatrophin and BMI and the waist-to-hip ratio (Zhang 2012; Abu-Farha et al. 2015). In another study, betatrophin levels were found to be elevated in obese subjects but decreased after exercise (Abu-Farha et al. 2016a).

Other concomitant diseases that may affect betatrophin levels may explain some of the discrepancies in reported betatrophin levels. For example, thyroid function, polycystic ovary syndrome, and metabolic syndrome have been associated with betatrophin levels. It was recently reported that betatrophin levels were higher in individuals with metabolic syndrome than healthy controls. When stratified by the number of metabolic syndrome components, people with more metabolic syndrome components had significantly higher betatrophin levels (Abu-Farha et al. 2016b). In addition, Crujeiras et al. found that obese patients with metabolic syndrome had higher betatrophin levels, which was associated with less weight loss and dyslipidemia after a weight loss program (Crujeiras et al. 2016).

A meta-analysis conducted by Li et al. has shown that betatrophin levels are associated with type 2 diabetes. This meta-analysis included nine studies that met their criteria. Nonobese diabetics with type 2 diabetes had higher betatrophin levels than nonobese nondiabetics. Comparing obese subjects with type 2 diabetes to obese nondiabetics, there was no significant difference in serum betatrophin levels. To be clear, their meta-analysis did not include any of the more recent data showing that betatrophin levels are higher in patients with type 2 diabetes (Li et al. 2016). The newer data had a much larger sample size than previous studies. For this reason, their meta-analysis referred to the presence of a small sample size as a primary limitation of a study when interpreting the results. Also, they noticed that betatrophin levels were found to vary widely in different studies because of the use of different ELISA kits and different betatrophin concentrations (Li et al. 2016).

Mini Dictionary of Terms

- Adipokines: They are cell-signaling proteins that are secreted by adipose tissue.
- Batokines: Adipokines that are derived from brown adipose tissue.
- Homeostasis model assessment of insulin resistance value: A method that measures insulin resistance and beta-cell function.
- Insulin resistance: Is a condition in which cells do not respond appropriately to the insulin hormone.
- db/db mouse: A mutant mouse that has homozygous diabetes spontaneous mutation causes obesity, chronic hyperglycemia, pancreatic beta-cell atrophy, and hypoinsulinemia,
- ob/ob mouse: A mutant mouse that overeats due to defects in the gene that produces leptin, resulting in extreme weight gain.

Key Facts of Betatrophin

- According to the most recent International Diabetes Federation data, gestational diabetes mellitus affects 16.7% of all pregnant women, resulting in approximately 21.1 million babies are born to pregnant women with diabetes each year.
- There are many factors that contribute to the development of gestational diabetes, but the most common is beta-cell dysfunction and increased insulin resistance throughout pregnancy.
- Betatrophin enhances pancreatic beta-cell replication.
- Betatrophin can inhibit the activity of lipoprotein lipase.
- The effect of betatrophin on human beta cell proliferation is still unknown.
- In recent years, there has been a rise in interest in assessing betatrophin circulating concentrations in patients as part of efforts to elucidate its putative function in metabolic diseases, including obesity, type 1, and type 2 diabetes.

Summary Points

- Insulin sensitivity changes during pregnancy due to variations in maternal hormones to accommodate the additional demands of the growing fetus.
- Betatrophin levels have been shown to be elevated in pregnant women with gestational diabetes mellitus in both maternal serum and cord blood.
- Certain differences exist between studies, which are most likely due to the small sample sizes and the wide range of detection kits used to test for betatrophin levels.
- Betatrophin is a promising diagnostic marker for gestational diabetes mellitus.
- Further research could help us better understand the role of betatrophin in the pathophysiology of gestational diabetes mellitus.

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Biomarkers in Disease: Diabetes Methods, Discoveries, and Applications

18

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Contents

Introduction 397
Risk Prediction of Diabetes and the Role of Lipoproteins 398
The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Results from Clinical Studies 398
The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Rationale Suggesting a Causal Role 399
The Role of VLDL Particles and Triglycerides in Risk Prediction of Diabetes: What is the Role of Genetics? 400
The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Genetics as a Fundamental Player 401
Lipidomics as Tool to Study TRL Metabolism and the Risk of Diabetes 402
Applications to Other Diseases or Conditions 404
Mini-Dictionary of Terms 404
Key Facts of Type 2 Diabetes and Hypertriglyceridemia 405
Summary Points 405
References 405

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Abstract

Triacylglycerol (TG)-enriched remnant lipoproteins (TRL) metabolism has a central role in cardiovascular disease, but more recently TRL particles got into evidence for their potential relationship with type 2 diabetes. TRL particle size and VLDL-triglyceride content have been implicated in the risk of type 2 diabetes and particularly a shift to larger VLDL particles (VLDL1) can improve risk prediction of type 2 diabetes. The chapter goes on to discuss research in humans that addresses these relationships, as well as potential direct and indirect mechanisms that may justify the use of TG-enriched remnant lipoprotein to predict future risk of type 2 diabetes. Obesity, insulin resistance and systemic inflammation play a role in increasing hepatic fat content and upregulating TRL particle size; however, it is less clear weather the connection VLDL1 and type 2 diabetes is direct (causal) or genetically mediated. Finally, the chapter discusses new perspectives in identifying new TG and TRL size-related biomarkers for the risk of type 2 diabetes and cardiovascular diseases, including untargeted lipidomics.

Keywords

Triacylglycerol-enriched remnant lipoproteins · TRL · TRL particle size · Triacylglycerol · Type 2 diabetes · VLDL1 · Cardiovascular diseases · Lipidomics · NMR · Mass spectrometry · Risk prediction

Abbreviations

ANGPTL3	Anti-angiopoietin-like protein 3
ApoAV	Apolipoprotein AV
ApoC3	Apolipoprotein C3
AUROC	Area under the receiver-operating characteristic curve
CV	Cardiovascular
CVD	Cardiovascular disease
DGAT1	Diacylglycerol O-Acyltransferase 1
ELSA-Brasil	Adult Health Logitudinal Study (*Estudo Longitudinal de Saúde do Adulto*) - Brazil
EPA	Eicosapentaenoic acid
Gpihbp1	Glycosylphosphatidylinositol-anchored HDL binding protein-1
GWAS	Genome-wide association studies
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment for insulin resistance
LDL	Low-density lipoprotein
LDL	Low-density lipoprotein cholesterol
LPL	Lipoprotein lipase
MTP	Microsomal triglyceride transfer protein
NMR	Nuclear magnetic resonance

SNP	Single nucleotide polymorphism
Srebp-1c	Sterol regulatory element-binding protein 1c
T2DM	Type 2 diabetes mellitus
TG	Triacylglycerol
TRL	Triacylglycerol-enriched remnant lipoproteins (TRL)
TNFα	Tumor necrose factor α
VLDL	Very low-density lipoprotein

Introduction

Clinical interest in the treatment of triacylglycerol (TG)-enriched remnant lipoproteins (TRL) has fluctuated between opposite extremes in recent decades. The hesitation between measuring and treating or not high levels of TRL has been driven by polarized evidence, sometimes suggesting that these lipoproteins may be causally associated with diabetes and atherosclerotic cardiovascular disease (CVD), and sometimes suggesting that such lipoproteins would represent only innocent bystanders of metabolism of high-density lipoproteins (HDL) (Chapman et al. 2011). Probably more important has been the stagnation on the basis of new clinical evidence or new specific therapies that could reinforce the benefit of reducing triglycerides. In addition, the measurement of TG-enriched remnant lipoproteins features other than TG content such as particle size, particle number and cholesterol content are still not commercially available in many countries.

In the 1980s and 1990s, most clinical guidelines suggested measuring and treating mild to moderately high triglyceride levels (200–500 mg/dL) (Pyorala et al. 1994). The wide interest in triglyceride-rich lipoproteins at the time was related to several factors, among them positive results from incipient clinical trials and the Zilversmith hypothesis (Zilversmit 1979), which explained atherogenesis and other metabolic diseases as a postprandial phenomenon and related to the increase in TG-enriched remnant lipoproteins.

However, from the late 1990s to recent years, most recommendations focused mainly on LDL (low-density lipoprotein) cholesterol and largely ignored the need for treatment of mild to moderately high levels of triglycerides. One of the main explanations for the lack of interest in TG-enriched remnant lipoproteins was the failure of most clinical trials with fibrates, which was interpreted by most clinicians as the failure of the "triglyceride hypothesis" (Group et al. 2014; Investigators et al. 2011). However, these clinical trials have not evaluated the effect of specific reduction of TG-enriched remnant lipoproteins on the risk diabetes.

In the last decade, the spotlight turned back to TG-enriched remnant lipoproteins, with the emergence of technologies combining niacin and EPA (CAT-2003), high-dose EPA (icosapent-ethyl), anti-apoC-III antisense therapies (volanesorsen), DGAT1 inhibitors (pradigastat), anti-angiopoietin-like protein 3 (ANGPTL3) antibodies (evinacumab), MTP inhibitors (lomitapide), LPL gene therapy (alipogene tiparvovec), and many others. With that, several novel population studies in parallel revealed TG-enriched remnant lipoproteins features showed strong association with

the risk of diabetes and CVD. In this context, and with the evidence from genetic studies accumulated in the last 10 years, TG-enriched remnant lipoproteins are once again gaining importance in the scenario of diabetes and CVD risk prediction.

Risk Prediction of Diabetes and the Role of Lipoproteins

Glycemic status at baseline is the most important predictor for future diabetes and individuals characterized by prediabetes show a 2- to 10-fold higher risk for diabetes compared to non-prediabetic individuals (Richter et al. 2018). Conversely, among those without pre-diabetes, the glycemic status has a smaller value and thus long-term risk prediction of diabetes in this population is much less precise (Abdul-Ghani and DeFronzo 2009). With these evidences, it becomes clear that new predictors are necessary to improve the capacity of identifying individuals at high risk of developing type 2 diabetes, especially among individuals without pre-diabetes.

Lipoproteins are key factor in trafficking lipids among tissues and particularly LDL and VLDL particles are implicated in the trafficking of lipids to pancreatic beta cells. Beta cell dysfunction is influenced by increased fatty acid content and increased plasma levels of both VLDL-triglycerides and LDL-cholesterol (Hao et al. 2007; Oh et al. 2018). Large VLDL particles usually carry an elevated content of triglycerides and animals with this phenotype show increased pancreatic cell lipotoxicity (Zhang et al. 2019). Pancreatic cell lipotoxicity is a major driver of beta cell dysfunction, thus increasing the risk of type 2 diabetes (Robertson et al. 2004). More recently, TRL particle size and VLDL-triglyceride content have been implicated in the risk of type 2 diabetes and particularly a shift from to high VLDL1 concentration with elevated TRL particle size can improve risk prediction of type 2 diabetes in individuals without pre-diabetes (Carvalho et al. 2021).

The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Results from Clinical Studies

The ELSA-Brasil study showed that, when participants without diabetes at baseline were divided in quartiles of TRL particle diameter, those larger TRL particles showed dysfunctional glucose homeostasis and lipid metabolism (Carvalho et al. 2021). Individuals with baseline HbA1c between 39 and 46 mmol/mol (5.7–6.4%) had stronger associations with insulin resistance (HOMA-IR), waist circumference, and hsCRP as compared to those with baseline HbA1c <5.7%. The study therefore hypothesizes that individuals with pre-diabetes status, baseline HbA1c of 5.7–6.4%, could increase the hepatic release of large VLDL particles or impair their catabolism (i.e., reduced lipoprotein lipase activity) due to a number of risk factors that they present, like obesity, hepatic steatosis, insulin resistance, and heightened inflammation (Carvalho et al. 2021).

Among subjects with intermediate hyperglycemia at baseline, TRL particle size present a borderline capacity for predicting diabetes when compared to a baseline model composed by age, sex, hypertension, parental history of type 2 diabetes, waist

circumference, systolic blood pressure, HbA1c, triacylglycerol and HDL-cholesterol. The baseline model shows area under the receiver-operating characteristic curve (AUROC) of 0.708 [95% CI 0.685, 0.732], while the addition of TRL particle size slightly increases AUROC to 0.729 [95%CI 0.696, 0.768] (p for difference = 0.0647). In multivariate analyses, the study found an increase for incident diabetes by 32% in each 10 nm increment in TRLZ size (95% CI 7.3, 57.2, $p = 0.0103$) (Carvalho et al. 2021).

Contrarywise, TRL particle size showed the best association with diabetes risk in subjects with no glycemic abnormalities at baseline. Furthermore, TRL particle size increased the AUROC for predicting type 2 diabetes on top of the best available model in a large and multi-ethnic cohort with a sustained univariate and multivariate effect.

Although it is clear that conditions such as obesity, insulin resistance and systemic inflammation play a role in increasing hepatic fat content and upregulating TRL particle size, it is less clear weather the connection between larger VLDL particles (VLDL1) and type 2 diabetes is direct (causal) or indirect through genetic mediation. In the next paragraphs the focus is to discuss the evidence for direct or indirect associations between TRL particle size and type 2 diabetes.

The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Rationale Suggesting a Causal Role

Inflammation stands as a critical player in the development of insulin resistance by affecting lipid metabolism in adipose tissues. Particularly, tumor necrosis factor (TNF)-α pathways inhibit lipoprotein lipase in adipose cells, resulting in increased non-esterified fatty acid level and causing overproduction of intestinally derived VLDL1 by stimuli of p38-mitogen-activated protein kinase (Akash et al. 2018; Qin et al. 2007). Furthermore, TNF-α active transcriptional factor, NF-kB, actively mediate the apoptosis of pancreatic ß-cells (Akash et al. 2018). Bearing these data, several anti-TNF-α treatments have been tested targeting insulin resistance mitigation and pancreatic ß-cell preservation. In experimental animal studies by Borst et al. (Borst and Bagby 2002), TNF-α inhibition improved insulin resistance, while the neutralization of TNF-α in an animal model by Tzanavari et al. (2010) improved the hepatic insulin resistance. In humans, Martin-Rodriguez et al. (2019) analyzed a healthy non-diabetic Caucasian population and showed a positive correlation between HOMA-IR and hepatic triglycerides, and also between plasma TNF-α concentrations and intrahepatic triglyceride content levels and HOMA-IR.

Insulin resistance is associated with increased production of glycerol 3-phosphate via increased gluconeogenesis and triglyceride synthesis via *de novo* lipogenesis. Both mechanisms trigger the expression of sterol regulatory element binding protein 1c ($SREBP_{1c}$) (Browning and Horton 2004). The overlap between variations in metabolism and the change in TRL physiology in insulin resistance could clarify the reason why TRL particle size augment the predictive capacity for type 2 diabetes as compared to classic risk factors in non-prediabetic subjects. (Carvalho et al. 2021).

Many studies have suggested a potentially causal relationship between VLDL1 levels and the risk of diabetes (Qi et al. 2012). Another interesting finding is that the accumulation of fat in non-adipose tissue (e.g., liver and muscle) is highly associated with the risk of type 2 diabetes mellitus (Czech et al. 2013). All these effects are potentially due to the development of pancreatic steatosis and cytotoxicity.

In mouse, Zhang et al (2019) chose two hypertriglyceridemia models: apolipoprotein C3-transgenic (ApoC3-tg) and glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein-1 knockout (Gpihbp1−/−). These genetic mutations are related with lipoprotein lipase activity, which leads to severe hypertriglyceridemia. Indeed, authors found that plasma from Gpihbp1−/− mice had much larger TRL particles, composed mainly of large chylomicron particles compared with ApoC3-tg mice, which has mainly small VLDL particles as adjusted by triglyceride content (Zhang et al. 2019). The pancreas of Gpihbp1−/− mice presented more free-fatty acids, hydrolysis products of TRLs, than ApoC3-tg mice suggesting that greater FFA production may predispose pancreatic necrosis, by cytotoxic effects in pancreatic acinar cells (Zhang et al. 2019).

Finally, several studies have demonstrated that decreasing the excess lipid from pancreas and liver improves insulin sensitivity, returning to a normal insulin secretion in early type 2 diabetes (Lim et al. 2011; Steven et al. 2016). Although these evidences are compelling to link TRL particle size to the risk of diabetes, they are not sufficient to state there is a causal role between these two elements. More recently, a large burden of evidence outlined a potential indirect role between TRL particle size and the risk of diabetes.

The Role of VLDL Particles and Triglycerides in Risk Prediction of Diabetes: What is the Role of Genetics?

The development of type 2 diabetes is a complex process resulting from the combination of genetic, environmental, behavioral, and aging factors. Family-based studies revealed that the complete understanding of the molecular mechanisms that are involved in the pathophysiology and susceptibility of type 2 diabetes is a challenge and is also influenced by genetic and epigenetic factors (Rich 1990). The risk of developing diabetes throughout lifespan is about 40% when one parent is affected, and this rate rises to 70% when both parents are diabetic (Rich 1990).

Genome-wide association studies (GWASs) were largely responsible for identifying more than 400 genetic variants at 250 distinct loci, including several single nucleotide polymorphisms (SNPs) associated with the risk of type 2 diabetes (Torres et al. 2014; Qi et al. 2012; Mahajan et al. 2018). These methods were able to identify genes associated with monogenic variants of type 2 diabetes such as lipodystrophy, severe insulin resistance (in non-obese individuals), and Maturity-Onset Diabetes of the Young (MODY). However, when the same methods were used to identify the most common forms of diabetes, only a few genes were shown as relevant (Billings and Florez 2010).

Despite GWASs have discovered many new genetic associations, these findings represent events of mid-frequency and common occurrences, not including rare frequency variants and minor variants of cumulative effect (Korte and Farlow 2013) {Billings and Florez 2010 #50}. Therefore, it is necessary to identify a part of the genetic variation that cannot be explained by all significant SNPs (Torres et al. 2014).

In an analysis focusing on the impact that the various polymorphisms have on the development of type 2 diabetes, a genotypic score was established to estimate the genetic predisposition to dyslipidemia based on well-established SNPs located in 95 loci for blood lipids, 25 of which had a direct influence on triglycerides. Each additional risk allele in the genotype scores of HDL cholesterol or triglycerides was associated with 2–3% increased risk for type 2 diabetes. Added the effects, this triglyceride genotype score was able to explain about 10% of the variation of plasma TRL in the evaluated population (Qi et al. 2012; Teslovich et al. 2010).

Most genetic polymorphism sites related to type 2 diabetes were identified by GWASs based on case-control cohorts of European descendants (Andersen et al. 2016). Most extensive data available comes from studies with Caucasian population, including more than 300,000 individuals, where the presence of one particular minor allele linked to rs662799 SNP associates with a 0.25 mmol/l in plasma TG levels (Triglyceride Coronary Disease Genetics et al. 2010). In other populations such as Latin American populations, the role of genetic loci in plasma TG levels and VLDL particles look more evident.

Among various polymorphisms associated with increased risk of type 2 diabetes in Latin American population the SLC16A11 gene polymorphisms have major importance. SLC16A11 messenger RNA is expressed in the liver and it influences lipid metabolism, mainly by promoting an increase in intracellular triacylglycerol levels. In an analysis that included Latin Americans, especially Mexicans, individuals that carry the risk haplotype develop type 2 diabetes approximately 2 years earlier than not carries. Another interesting finding was that the same patient had a higher incidence of type 2 diabetes, even with a body mass index nearly 1 kg/m^2 lower and, finally, the odds ratio for type 2 diabetes among those with the risk haplotype was higher in younger individuals ($\leq$45 years) than older individuals (OR 1.48 vs. 1.11) compared to those individuals without the risk haplotype (Consortium et al. 2014).

The Role of Large VLDL Particles (High Density of VLDL1 Particles) in Risk Prediction of Diabetes: Genetics as a Fundamental Player

Due to the specificity of common single nucleotide variants among different ethnic groups that have already been subjected to genotyping, more specific variants have been identified, often with different phenotypic impacts in each ethnic group (Andersen et al. 2016). A well-documented SNP identified as rs12310367 in the apolipoprotein C3 (APOC3) is directly related to alterations in the production of the protein component of TRLs, including VLDL (Valladolid-Acebes et al. 2021).

Overexpression of APOC3 is associated with high triglyceride levels and large VLDL particles, while reduction or loss of function mutations in the gene encoding APOC3 are associated with low plasma triglyceride levels and reduced risk of ischemic cardiovascular disease (Adiels et al. 2019). In individuals with abdominal obesity, plasma triglyceride concentrations are elevated as well as the presence of VLDL1 subspecies. The catabolism of VLDL1 is mostly dependent on APOC3 concentration. Furthermore, overexpression of APOC3 has been related to the promotion of VLDL assembly and secretion in hepatocytes in genetically modified mice in vitro experiments (Boren et al. 2015).

In addition to these findings, it has been reported that APOC3 metabolism is significantly unsettled in type 2 diabetes patients. These evidences suggest that glucose homeostasis is associated with APOC3 metabolism, and that the secretion rate of APOC3 seems to be a relevant actor in increasing TRL particle size and triglyceride content observed among individuals with type 2 diabetes (Boren et al. 2020).

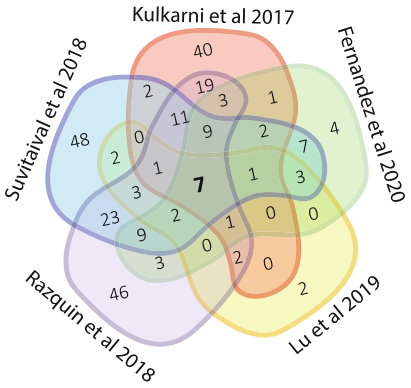
Finally, apolipoprotein AV (ApoAV) is also associated with TG-rich lipoproteins. APOAV gene and its genetic variants influence both plasma triglyceride levels and VLDL remodeling. In addition, ApoAV also reduces plasma triglycerides by inhibiting VLDL-TG production and stimulating lipoprotein lipase-mediated VLDL-TG hydrolysis, thus reducing VLDL particle size (Schaap et al. 2004). Two minor alleles (C1131 and Trp19) of APOAV are related to reduced activity of APOAV and results in high plasma triglyceride levels (Hubacek 2016).

These genetic polymorphisms could underlie the indirect relationship between TRL particle size and the risk of type 2 diabetes. However, as pointed out, the genetic background does not fully answer this link and it is plausible to consider that both direct and indirect effects play a role. To better understand pathways to identify more specific markers to link TRL particle size and the risk of diabetes, the next section is dedicated to discuss the perspective of using lipidomics to study TRL metabolism and its associations with diseases.

Lipidomics as Tool to Study TRL Metabolism and the Risk of Diabetes

Advances in liquid chromatography tandem mass spectrometry (LC-MS) technologies have enabled an initial characterization of the human plasma lipidome (i.e., lipidomics (Quehenberger et al. 2010, 2011; Bowden et al. 2017)) and thereby its application to the discovery of novel lipid markers and targets for treatment of metabolic diseases (Rhee et al. 2011; Stegemann et al. 2014; O'Donnell et al. 2014). A direct link between TRL and lipidomics is to ascertain specific TG species as markers of health and disease status. The importance of such data relies on the composition of specific fatty acids esterified to TG, allowing a categorization of plasma TG species into different fatty acids (i.e., short, medium, long, and very long chains or degrees of unsaturation). This information could provide essential clues on their association with and effects on blood lipids, glucose-insulin homeostasis, insulin resistance, and diabetes.

A.



B.

Lipid species associated with T2DM incidence

	all	4 out of 5	3 out of 5
# lipids	7	21	54
% TG	100%	81%	59%
TG-FA	16.7:0.7	16.9:0.7	17.1:0.8
TG sp.	TG (48:1) TG (48:2) TG (50:1) TG (50:2) TG (50:3) TG (51:2) TG (54:3)	TG (48:0) TG (48:3) TG (49:1) TG (50:0) TG (51:1) TG (51:3) TG (52:2) TG (53:2) TG (56:6)	TG (46:1) TG (46:2) TG (49:2) TG (50:4) TG (51:0) TG (52:1) TG (53:3) TG (54:1) TG (54:2) TG (54:4) TG (54:6) TG (54:7) TG (56:3) TG (56:4) TG (56:5)

Fig. 1 Comparative analysis of five comprehensive lipidomic studies in the context of T2DM incidence. (**a**) Venn-diagram displaying the number of overlapping lipid species associated with T2DM incidence. (**b**) Detailed analysis of the number of lipids (#lipids) commonly associated with T2DM incidence to all studies, 4 or 3 out of 5 studies. Given that several lipid species belonged to TG (%TG), the average number of carbon atoms and double bonds in fatty acids linked to TG (TG-FA as X:Y, where X and Y denote the number of carbon atoms and double bonds, respectively) and the diagnostic TG species (TG sp. as X:Y) are shown (Kulkarni et al. 2017; Razquin et al. 2018; Suvitaival et al. 2018; Lu et al. 2019; Fernandez et al. 2020)

To illustrate the potential of lipidomics in predicting T2DM, a selected list of five recent papers involving a comprehensive analysis of the plasma lipidome was compared (Fig. 1). Although investigating an extensive panel of lipids (180–800 species divided into 8–24 lipid classes), these studies revealed a surprisingly low number of overlapping lipid species associated with the incidence of T2DM (Fig. 1a). Nonetheless, there is a consistent prevalence of TG species among plasma lipid classes from which concentrations are positively correlated with T2DM incidence (Fig. 1b), despite the distinct populations (e.g., METSIM, SAFHS, PREDIMED, and REACTION cohorts) and periods for T2DM incidence (ranging from 3.8 to 21.2 years) reported. The data also revealed that most of these specific TG species are linked to fatty acids containing low number of carbon atoms and double bonds, with an averaged chain length and unsaturation of ca. 17 carbons and 0.7 double bonds (Fig. 1b). While far from conclusive, the data in Fig. 1 suggest that the prevalence of such TG species characterizing cases of incident T2DM might be linked to TRL levels.

The assessment of TRL levels is challenging due to several factors including interindividual and intraindividual variation in plasma TG levels, highly heterogeneous size, density and composition of circulating TRL, and distinct sources of remnant lipoproteins (i.e., intestine or liver derived particles (Twickler et al. 2004)). The concentrations of TRL have been measured by different methods based on density, size, electrostatic charge, lipid and apolipoprotein components, or immunoaffinity (Cohn et al. 1999). For large-scale population studies, modern

methods to access TRL levels include ^{1}H-NMR lipoprotein spectroscopy analysis that can be applied to determine subclasses of TRL based on their size spectrum (Carvalho et al. 2021; Varga et al. 2021). The NMR is a low-cost and nondestructive technique that performs a quick analysis with highly reproducible data and free of batch analysis effects (i.e., no interaction of samples and equipment). While MS-based lipidomics is highly sensitive (can be used to monitor hundreds of compounds) and requires only a small volume of sample, there are some drawbacks in applying MS for population studies. For instance, the elevated costs associated with sample preparation (e.g., requirement for analytical internal standards to allow quantitative analysis), moderate data reproducibility and loss of sensitivity in batch analysis (due to variations in ionization process). Nonetheless, NMR lipoprotein analysis and MS-based lipidome profiling are rather complimentary (Emwas et al. 2019) and when combined may provide a powerful tool to discover new lipid markers and biological pathways relevant to TRL metabolism in a cost-effective manner. Such combined approach in large-scale studies would be ideally initiated by an NMR screening of all samples to identify divergent groups of patients, followed by a comprehensive and quantitative analysis by MS in a limited number of interesting and extreme samples. However, to advance into integrative approaches between lipidomics and other applied analytical methods (e.g., NMR or genomics), further improvements are still required, particularly concerning the standardization of methods for reporting quantitative and reproducible data (Bowden et al. 2017; Burla et al. 2018; O'Donnell et al. 2020).

Applications to Other Diseases or Conditions

In this study we review the use TRL particle size to predict the risk of type 2 diabetes and the potential mechanisms associated to this link. In summary, risk prediction of T2DM in primary prevention could be improved by using TRL particle size particularly among individuals with no abnormalities in glucose metabolism at baseline. Nonetheless, there are other potential applications for this biomarker. Of note, TRL particle size is also linked to the risk of myocardial infarction and peripheral artery disease (Duran et al. 2020), and play a major role in atherogenesis and chronic coronary artery disease (Twickler et al. 2004). Furthermore, the robust associations found between TRL particle size and the risk of type 2 diabetes offer a new perspective for the development of targeted interventions aiming to reduce TRL particle diameter and TG content per particle to control metabolic diseases.

Mini-Dictionary of Terms

- ***Type 2 diabetes*** *A chronic condition that affects the way the body processes blood sugar, due to both peripheral resistance to insulin action and dysfunctional production of insulin by pancreatic beta cells.*
- ***VLDL*** *Very low-density lipoprotein is produced in the liver and released into the bloodstream to supply body tissues with triglycerides.*

- ***Genetic polymorphism.*** *Defined as the inheritance of a trait controlled by a single genetic locus with two alleles, in which the least common allele has a frequency of about 1% or greater. Genetic polymorphism is a mutation in DNA sequence among individuals, groups, or populations.*
- ***GWAS*** *A genome-wide association study is an observational study of a genome-wide set of genetic variants in different individuals to see if any variant is associated with a trait/condition.*
- ***^{1}H-NMR spectroscopy*** *Proton NMR is a powerful tool for molecular structure characterization.*

Key Facts of Type 2 Diabetes and Hypertriglyceridemia

In the last 20 years, the number of adults diagnosed with diabetes has more than doubled as the world population has aged and become more overweight or obese

Diabetes is the 7th leading cause of death in the United States

At least 60% of individuals with type 2 diabetes show hypertriglyceridemia and other types dyslipidemia

Lipoproteins such as HDL-C, LDL-C and VLDL-C are implicated as risk factors for type 2 diabetes

Increased lipid content in pancreatic beta cells may induce lipotoxicity and apoptosis, leading to insulin secretory dysfunction.

Summary Points

- *TRL particle size and VLDL-triglyceride content have been implicated in the risk of type 2 diabetes*
- *Particularly a shift to larger VLDL particles (VLDL1) can improve risk prediction of type 2 diabetes*
- *Obesity, insulin resistance, and systemic inflammation play a role in increasing hepatic fat content and upregulating TRL particle size*
- *Genetic polymorphisms could explain both increased TRL particle size and the risk of type 2 diabetes*
- *Data from mass spectrometry studies suggest TG species characterizing cases of incident T2DM might be linked to TRL levels*

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Correlations Between Renal Biomarkers and the Treatment Outcomes in Diabetes: Ophthalmic Aspects

19

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Contents

Introduction 413
Correlations Between Renal Biomarkers and Diabetic Retinopathy 413
Serum Creatinine and Estimated Glomerular Filtration Rate 413
Albuminuria 414
Erythropoietin 414
Correlations Between Renal Biomarkers and Diabetic Macular Edema 415
Serum Creatinine and Estimated Glomerular Filtration Rate 415
Albuminuria 415
Body Fluid Status 416
Correlations Between Renal Biomarkers and Treatment Outcomes of Diabetic Macular Edema 416
Serum Creatinine and Estimated Glomerular Filtration Rate 416
Albuminuria 416
Effects of Diabetic Nephropathy Treatment on Diabetic Retinopathy and Diabetic Macular Edema 417
Glycemic Control 417
Glucagon-Like Peptide-1 (GLP-1) Agonists 418
Dipeptidyl Peptidase-4 (DPP-4) Inhibitors 420
Sodium-Glucose Co-Transporter-2 (SGLT-2) Inhibitors 420
Renal Replacement Therapy 420
Kidney Transplantation 421
Correlations Between Renal Biomarkers and Other Ocular Manifestations in Diabetes 421
Diabetic Keratopathy and Corneal Nerve Degeneration 421
Renal Biomarkers Versus Diabetic Keratopathy 421

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Effects of Renal Replacement Therapy or Kidney Transplantation on Diabetic Keratopathy 422
Glaucoma in Diabetes 422
Renal Biomarkers Versus Glaucoma in Diabetes 423
Effects of Renal Replacement Therapy or Kidney Transplantation on Glaucoma 423
Renal Biomarkers Versus Cataract in Diabetes 423
Effects of Renal Replacement Therapy or Kidney Transplantation on Cataract 424
Conclusions 424
Applications of Renal Biomarkers to Prognosis 424
Mini-dictionary of Terms 425
Key Facts of Diabetic Retinopathy 425
Key Facts of Diabetic Macular Edema 426
Summary Points 426
References 426

Abstract

Diabetic retinopathy (DR) and diabetic nephropathy are the main microvascular complications in diabetes and often comorbid with each other. Renal biomarkers including serum creatinine, estimated glomerular filtration rate (eGFR), and urine albumin-to-creatinine ratio (UACR) are all associated with the incidence and severity of DR; better control of these renal biomarkers may help slow down the progression of DR. Diabetic macular edema (DME) is the leading cause of blindness resulting from DR; the main treatment is intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents nowadays. Patients with low eGFR may have poorer visual outcome after anti-VEGF treatment for DME. Patients with albuminuria tend to have more severe serous-type macular edema at baseline, but respond well to anti-VEGF treatment. Hemodialysis is helpful for controlling macular edema in patients with end-stage renal diseases, but it might aggravate diabetic keratopathy and increase the risk of glaucoma. Renal transplant may be helpful for DR, DME, diabetic keratopathy, and glaucoma.

Keywords

Diabetic retinopathy · Diabetic macular edema · Diabetic nephropathy · Diabetic keratopathy · Glaucoma · Cataract · Glomerular filtration rate · Proteinuria · Albuminuria

Abbreviations

DKD	Diabetic kidney disease
DME	Diabetic macular edema
DN	Diabetic nephropathy
DPP-4	Dipeptidyl peptidase-4
DR	Diabetic retinopathy
eGFR	Estimated glomerular filtration rate
GLP-1	Glucagon-like peptide-1
HbA1c	Glycated hemoglobin

IOP	Intraocular pressure
NPDR	Nonproliferative diabetic retinopathy
PDR	Proliferative diabetic retinopathy
SGLT-2	Sodium-glucose co-transporter-2
STDR	Sight-threatening diabetic retinopathy
T1D	Type 1 diabetes
T2D	Type 2 diabetes
UACR	Urinary albumin creatinine ratio
VEGF	Vascular endothelial growth factor

Introduction

Diabetic retinopathy (DR) is one of the main microvascular complications of diabetes. Numerous studies have demonstrated that diabetic nephropathy (DN), or diabetic kidney disease (DKD), is often comorbid with DR (Pavkov et al. 2019; Hsing et al. 2020; Park et al. 2019; Lee et al. 2014; Pedro et al. 2010; Wong et al. 2008; Wang et al. 2020a). It is of no wonder that these two microvascular complications of diabetes happen together. Therefore, renal biomarkers might be related to the development and the treatment effect of DR. Furthermore, other ocular manifestations may also happen in diabetic patients, including diabetic keratopathy, glaucoma, and cataract. In this chapter, we will review the evidences of correlations between renal biomarkers and the diabetic eye disease.

Correlations Between Renal Biomarkers and Diabetic Retinopathy

The comorbidity of DR and DN has been well known, and DN is definitely an important risk factor for the development and progression of DR. An 8-year prospective cohort study of patients with type 2 diabetes (T2D) in Taiwan revealed that abnormal renal profiles at baseline and during follow-up were both associated with the development of proliferative diabetic retinopathy (PDR) (Hsieh et al. 2018). Another longitudinal study from India also demonstrated similar results that DKD with reduced estimated glomerular filtration rate (eGFR) or presence of albuminuria was correlated with the development of sight threatening DR (STDR) (Rajalakshmi et al. 2020). One study evaluated the scores of interstitial fibrosis and tubular atrophy (IFTA) through renal biopsy (Yamanouchi et al. 2019) and found that the IFTA score was correlated with DR severity scales ($r = 0.41$) (Wilkinson et al. 2003). The correlations between common renal biomarkers and DR will be discussed in details followingly.

Serum Creatinine and Estimated Glomerular Filtration Rate

Serum creatinine was found to be higher in subjects with DR than those without (Mathala et al. 2020). A higher level of serum creatinine was associated with the

development of PDR (Hsieh et al. 2018) and disruption of photoreceptors (Saxena et al. 2017) in T2D. More studies have revealed the correlations between estimated glomerular filtration rate (eGFR) and DR. A low eGFR has been linked to the development or progression of DR in numerous studies (Hsieh et al. 2018; Man et al. 2015; Kaewput et al. 2019). However, early DR can still develop in patients with normal eGFR (Wu et al. 2015). In this study it was observed that an eGFR of 99.4 mL/min/1.73 m^2 or less in T2D might indicate the early stage of DR. This could be in part attributed to the phenomenon of glomerular hyperfiltration, which leads to an increase in eGFR in the early stage of diabetes (Tonneijck et al. 2017). Overtime, the hyperfiltration results in irreversible renal damage and further decline in glomerular filtration rates. The pathogenesis for glomerular hyperfiltration is complex. However, it sheds light on some inconsistent clinical findings about the associations between renal function and DR (Sabanayagam et al. 2014; Penno et al. 2012).

Albuminuria

Albuminuria is the most frequently used biomarker for DN in current literatures. Microalbuminuria has been found to be associated with the development and severity of DR in T2D patients (Hsieh et al. 2018; Manaviat et al. 2004; Lee et al. 2017; Reddy et al. 2013). Some studies suggest that microalbuminuria is a more sensitive biomarker than reduced eGFR with regard to the prediction of DR progression (Romero-Aroca et al. 2018; Chen et al. 2012). Although chronic hyperglycemia is the trigger factor for subsequent cascades resulting in both disruption of glomerular filtration barrier and blood–retinal barrier in diabetic patients, whether these events occur simultaneously remains unclear. Remission of microalbuminuria was shown to be protective for the development of PDR, while the development of DR or PDR did not result in progression of albuminuria (Hsieh and Hsieh 2021). Such time-sequential relationship between albuminuria and DR suggests that VEGF might be expressed and released into systemic circulation after glomerular injury, and then further affect the retina (Cha et al. 2004; Pawlak et al. 2008).

Erythropoietin

Reduced erythropoietin may be an indicator for early DN (Bosman et al. 2001). It is one of the various causes of anemia, which has been regarded as a risk factor for DR and DME (Wang et al. 2020a; McGill and Bell 2006). However, the role of erythropoietin is complex. Whether it is beneficial or detrimental to DR remains undetermined (Reid and Lois 2017; Davidović et al. 2019; Semeraro et al. 2014). Although once thought to be secreted by kidney in early studies, erythropoietin can also be produced in response to tissue hypoxia in many tissues including retina, and is no longer considered a specific biomarker for renal function.

Correlations Between Renal Biomarkers and Diabetic Macular Edema

Diabetic macular edema (DME) is the main cause of visual loss in patients with DR, and it is caused by increased permeability of macular retinal vessels and exudation of serous fluid and lipids into the macula. Since DME can occur at any stage of DR, other mechanisms could possibly be involved in the formation of macular edema and share the routes of pathogenesis with DR. Therefore, the renal biomarkers for DME might not be exactly the same as the biomarkers for DR.

Serum Creatinine and Estimated Glomerular Filtration Rate

While the eGFR level is correlated with the DR severity scale, the associations between eGFR and DME do not appear consistent in current studies (Hsieh et al. 2018; Man et al. 2015; Romero-Aroca et al. 2018; Temkar et al. 2018; Zhuang et al. 2019; Peng and Tsai 2018). Disparate analytical methods could be one of the reasons for such inconsistency. For example, in the study by Romero-Aroca et al. (2018), the eGFR levels were significantly higher in patients with DME or STDR than those with any DR. However, in multivariate analysis, the eGFR was no longer significantly correlated with DME or STDR. Similarly, Zhuang et al. showed that eGFR was a marker for DME in univariate analysis, but not in the multivariate models (Zhuang et al. 2019). Man et al. also found that a lower eGFR was correlated with more severe DR but not with DME, even after adjustment for essential baseline factors (Man et al. 2015).

Albuminuria

In contrast to eGFR, UACR has been shown to be a more sensitive and consistent renal biomarker in predicting DME. The severity of albuminuria has been identified as a risk factor for DME (Hsieh et al. 2018; Zhuang et al. 2019). Observations from the clinical evidences suggest that disruption of the glomerular filtration barrier seems to occur in parallel with breakdown of the blood–retinal barrier, which is the key factor for DME. Microalbuminuria was an independent factor for the presence of hard exudate and clinically significant macular edema in T2D (Ajoy Mohan et al. 2011). Interestingly, one study also showed the association between albuminuria and the patterns of macular edema (Koo et al. 2013); the level of albuminuria was the highest in serous type of macular edema than other types including diffuse, cystoid, vitreomacular adhesion, or mixed types. Patients with the serous type of DME were also found to have lower serum albumin levels, probably as a result of renal dysfunction. Low serum albumin level leads to a decrease in serum osmotic pressure, which in turn may cause body fluid overload and serous edema. This could be further demonstrated from the reports that macular edema happened in cases of renal failure without diabetes (Williams et al. 2006). It is

inferred that systemic diuretics may play a role in the treatment for this type of edema (Ciardella 2004), while the effect needs to be confirmed in further clinical trials.

Body Fluid Status

Recently, volume expansion has been linked to the presence of macular edema in patients with DR (Tsai et al. 2019). This study examined the body fluid compartments and found that subjects with higher levels of extracellular fluid and overhydration were associated with the presence of macular edema, and that patients with the presence of both subretinal fluid and intraretinal cyst had the highest level of relative overhydration.

Correlations Between Renal Biomarkers and Treatment Outcomes of Diabetic Macular Edema

Serum Creatinine and Estimated Glomerular Filtration Rate

Persistent subretinal fluid following consecutive loading injections of ranibizumab for DME was more likely to be found in patients with a baseline eGFR less than 60 mL/min/1.73 m^2 (Tsai et al. 2017). Lai et al. (2020) found that patients with an eGFR less than 30 mL/min/1.73 m^2 tended to have poorer visual outcomes after 1 year of intravitreal ranibizumab injections, and Sophie et al. (2015) also confirmed that renal diseases predicted poor visual outcomes after 2-year ranibizumab treatment for DME in the RIDE and RISE clinical trials. However, another post hoc analysis from the same clinical trials revealed no correlations between serum creatinine or eGFR and visual outcomes (Singh et al. 2016), which should be related to different statistical methods used for post hoc analysis. While no data of macular perfusion were included in this study, previous researches have demonstrated that chronic kidney disease with a reduced eGFR level was correlated with larger retinal capillary nonperfusion areas and lower vessel density at macula (Min et al. 2020; Wang et al. 2020b).

Albuminuria

For patients receiving ranibizumab for DME, those with more severe proteinuria had thicker central subfield thickness at baseline, but responded better than those with normal or only mild proteinuria (Lai et al. 2020). In the post hoc analysis of several clinical trials including RIDE/RISE (Sophie et al. 2015), DRCR.net Protocol I (Bressler et al. 2012), and protocol T (Bressler et al. 2019), serous-type edema was found to respond better to anti-VEGF therapy in terms of visual and anatomic outcomes compared to cystoid-type edema. Interestingly, it was patients with serous-type edema that were prone to albuminuria (Koo et al. 2013). The formation

of serous-type edema was blamed on the destruction of blood–retinal barrier and could be exacerbated by the decreased oncotic pressure and the increased hydrostatic pressure in the setting of albuminuria (Klaassen et al. 2013; Daruich et al. 2018). Anti-VEGF agents allowed the restoration of blood–retinal barrier and diminished such synergic effects, making body fluid status a predictor of the responsiveness to anti-VEGF therapy. The same concept could also account for the fact that, when treated with anti-VEGF in a pro re nata strategy, DME patients with more severe proteinuria or albuminuria required more frequent intravitreal injections of anti-VEGF agents during the first year (Lai et al. 2020; Liu et al. 2019). This means that anti-VEGF therapy is effective in blocking the extravasation from the leaking retinal capillaries in DME. However, once the drug effect resolves, the macula will become edematous soon due to the low oncotic pressure in patients with albuminuria, so these patients will need anti-VEGF treatment with shorter intervals for DME.

Effects of Diabetic Nephropathy Treatment on Diabetic Retinopathy and Diabetic Macular Edema

Similar to all diabetes-related complications, the basic treatment for diabetic nephropathy is glycemic control. Several kinds of new-generation oral hypoglycemic agents including glucagon-like peptide-1 (GLP-1) agonists, dipeptidyl peptidase-4 (DPP-4) inhibitors and sodium-glucose co-transporter-2 (SGLT-2) inhibitors are especially helpful in preserving kidney function. Renal replacement therapy and kidney transplantation are used for patients with end-stage renal diseases. Their effects on DR and DME will be discussed.

Glycemic Control

It has been well established that tight glycemic control can prevent the development and progression of DR. In the Diabetes Control and Complications Trial (DCCT), intensive glycemic therapy to keep the HbA1c level around 7% could prevent the occurrence of DR by 76%, slow the progression of existing DR by 54%, and reduce the development of PDR by 47% over 7 years in patients with T1D compared to those aimed at HbA1c levels of around 9% (Effect of intensive diabetes treatment 1994). The United Kingdom Prospective Diabetes Study (UKPDS), which recruited patients with new-onset T2D, also demonstrated that patients who maintained HbA1c levels of around 7% benefited from 21% reduction in risk for DR in 12 years (Intensive blood-glucose control 1998). In addition, the "metabolic memory" of early intensive glycemic control provided long-lasting protective effects against diabetic retinopathy despite increasing hyperglycemia in both of the studies (Barr 2000; Holman et al. 2008). However, tighter glucose control toward even lower HbA1c levels (<7%) did not show consistent benefit to DR across studies, but posed higher risk of severe hypoglycemic events (Group AS et al. 2010; Group AC et al. 2008; Lind et al. 2019). On the other hand, a higher

fluctuation of fasting glucose was found to be correlated with the development of PDR and DME (Hsieh and Hsieh 2020), indicating the importance of long-term stability in plasma glucose.

For the influence of glycemic control on the treatment outcomes of anti-VEGF therapy for DME, several studies have shown contradictory findings (Table 1). The post hoc analysis of DRCR.net protocol T and VIVID/VISTA clinical trials suggested that patients with a lower HbA1c level gained more visual improvement after treatment (Bressler et al. 2019; Singh et al. 2017), whereas such link was not seen in the DRCR.net protocol I and RISE/RIDE trials (Bressler et al. 2012; Bansal et al. 2015). On the contrary, although the aforementioned randomized controlled trials (RISE/RIDE, VISTA/VIVID, DRCR.net protocol I and protocol T) found no impact of HbA1c level on the anatomic outcome (Bressler et al. 2012, 2019; Singh et al. 2017; Bansal et al. 2015), some other prospective and retrospective studies demonstrated that patients with a lower HbA1c level had greater reduction of central foveal thickness but not better visual improvement (Ozturk et al. 2011; Wong et al. 2020; Sharma et al. 2020). Moreover, another two retrospective studies revealed that patients with a lower HbA1c level had thicker baseline central foveal thickness (Macky and Mahgoub 2013; Matsuda et al. 2014) but possessed better final vision. Such discordance between anatomical and visual outcomes is of no surprise, since central foveal thickness is not the only factor that determines visual acuity (Diabetic Retinopathy Clinical Research N et al. 2007). The reason for the diverse results of the correlations between glycemic control and treatment outcomes for DME is that hypoglycemia itself may be harmful. Several studies have revealed the U-shaped relationship between HbA1c level and cardiovascular diseases, stroke or all-cause mortality in diabetic patients (Anyanwagu et al. 2019; McAlister et al. 2020; Shen et al. 2020). Further studies are needed to clarify the role of HbA1c in its influence on anti-VEGF therapy for DME.

Glucagon-Like Peptide-1 (GLP-1) Agonists

A retrospective study found that GLP-1 agonist was capable of preventing non-proliferative diabetic retinopathy (NPDR) from evolving into STDR (Tauqeer et al. 2021). On the other hand, another retrospective study found that 30% of the patients treated with exenatide for 10 months experienced DR progression, among whom 80% of the cases had improved DR with sustained treatment (Varadhan et al. 2014). Similarly, the incidence of DR was higher for patients receiving GLP-1 agonist such as semaglutide or liraglutide in randomized trials evaluating their cardiovascular outcomes (Marso et al. 2016a, b). In response to these results, which was not seen in the SUSTAIN 1-5 trials for semaglutide, a post hoc analysis of the SUSTAIN 6 trial figured out that the worsening of DR was largely blamed on the drastic reduction of HbA1c in patients initially with preexisting DR and poor glycemic control (Vilsboll et al. 2018). Experimental studies on animal models have demonstrated that intravitreal or systemic GLP-1 agonists avoid nerve damage by preventing the activation of glial cells and maintaining the integrity of blood–retinal barrier (Pang

Table 1 Summary of studies evaluating the influence of glycemic control on the anti-VEGF therapy for DME. Most studies adopted baseline HbA1c as a parameter and attempted to find its correlations with visual and anatomic characteristics at baseline and after anti-VEGF treatment. The results were inconsistent across the studies

			Baseline HbA1c<7						Change in HbA1c during follow-up period		
Study	Design	Follow-up period	Baseline CRT	Final CRT	Change in CRT	Baseline VA	Final VA	Change in VA	Final CRT	Final VA	Reference
RISE/ RIDE	RCT	36 months	Thicker	Thicker	N/R	N/R	N/R	N/R	N/R	N/R	(Bansal et al. 2015)
VISTA/ VIVID	RCT	100 weeks	–	–	N/R	–	–	Better	–	–	(Singh et al. 2017)
DRCR I	RCT	12 months	–	–	N/R	–	–	N/R	–	–	(Bressler et al. 2012)
DRCR T	RCT	24 months	–	–	N/R	–	–	Better	–	–	(Bressler et al. 2019)
Warid et al.	Prospective	3 months	–	–	N/R	–	–	Better	–	–	(Warid Al-Laftah et al. 2010)
Ozturk et al.	Retrospective	4–6 weeks	–	–	Better	–	–	N/R	–	–	(Ozturk et al. 2011)
Macky et al.	Retrospective	6 months	Thicker	N/R	–	Better	Better	–	N/R	N/R	(Macky and Mahgoub 2013)
Matsuda et al.	Retrospective	12 months	Thicker	N/R	N/R	N/R	Better	N/R	–	–	(Matsuda et al. 2014)
Wong et al.	Prospective	12 months	N/R	–	Better	–	–	N/R	–	–	(Wong et al. 2020)
Sharma et al.	Prospective	3 months	–	–	Better	–	–	N/R	–	–	(Sharma et al. 2020)

CRT central retinal thickness, *N/R* no significant relevance, *RCT* randomized controlled trial, *VA* visual acuity

et al. 2018). Above all, despite the potential beneficial effect of GLP-1 agonist on retina, further clinical trials are needed to elucidate the impact of GLP-1 agonist on DR or DME.

Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

Experimental studies have revealed the direct potential beneficial effects of DPP-4 inhibitors on both DN and DR (Avogaro and Fadini 2014). A double-blind randomized controlled trial showed that saxagliptin could reduce hyperperfusion of retinal capillaries, which was an early sign of DR (Ott et al. 2014). A retrospective study (Kolaczynski et al. 2016) showed that treatment with vildagliptin was associated with reduced incidences of both DN and DR compared to treatment with sulfonylurea. However, another retrospective study (Kang et al. 2021) showed that add-on DPP-4 inhibitor therapy may be associated with DR progression in patients with T2D. A post hoc analysis of VISTA and VIVID trials showed that DPP-4 inhibitor use at baseline did not influence the magnitude of visual or anatomic improvement in patients receiving aflibercept or laser treatment for DME (Rahimy et al. 2020). Further studies are needed to reveal the effect of DPP-4 inhibitors on DR progression or anti-VEGF treatment for DME.

Sodium-Glucose Co-Transporter-2 (SGLT-2) Inhibitors

A double-blind randomized controlled trial showed that dapagliflozin could reduce hyperperfusion of retinal capillaries and minimize arteriole remodeling, both of which were early signs of DR (Ott et al. 2017). Some case reports also showed that SGLT-2 inhibitors were effective in treating DME (Yoshizumi et al. 2018; Takatsuna et al. 2020). However, the "beyond blood glucose control" effect of SGLT2 inhibitors on DR or DME merits further studies.

Renal Replacement Therapy

The effect of hemodialysis on DME has remained inconclusive according current evidences (Takamura et al. 2020; Tokuyama et al. 2000; Theodossiadis et al. 2012; Kumar et al. 2021; Azem et al. 2014; Mirza et al. 2013; Matsuo 2006). While an early report demonstrated no significant improvement of fluorescein leakage after hemodialysis (Tokuyama et al. 2000), some recent studies using optical coherence tomography (OCT) revealed reduction of retinal and choroidal thickness after hemodialysis (Takamura et al. 2020; Theodossiadis et al. 2012; Mirza et al. 2013). The mechanisms for this dynamic change was not clear; but it was believed to be related to the alteration of intravascular osmotic pressure caused by hemodialysis and subsequent decrease in extracellular fluid accumulation. Among different types of macular edema, subretinal fluid was the most responsive to

hemodialysis, compared to cystoid macular edema or sponge-like swelling (Takamura et al. 2020). Data regarding peritoneal dialysis for macular edema was limited. One case report revealed that initiation of peritoneal dialysis might contribute to the resolution of persistent edema under the treatment of intravitreal injections (Ong et al. 2017).

Kidney Transplantation

Kidney transplantation may play a role in stabilization of DR in a majority of patients who have already presented with varying degrees of DR at the time of transplantation (Mittal et al. 2005; Roy et al. 2013). According to one previous report (Roy et al. 2013), although early worsening of DR might be observed in some patients, most patients had their DR stabilized 75 months after the transplantation. Overall, 60% of patients had stable DR at final follow-up. Combined pancreas and kidney transplantations may be particularly helpful in stabilization of DR for patient with T1D (Chow et al. 1999; Pearce et al. 2000). Additionally, one clinical trial (Koznarová et al. 2000) found that patients with simultaneous pancreas and kidney transplantation received less additional laser treatment than kidney transplantation alone group in patients with T1D.

Correlations Between Renal Biomarkers and Other Ocular Manifestations in Diabetes

Diabetic Keratopathy and Corneal Nerve Degeneration

Diabetic keratopathy is also an ocular complication of diabetes (Markoulli et al. 2018). Under chronic hyperglycemia, progressive axonal degeneration of corneal nerve develops because advanced glycation end products accumulate around nerve tissues and cause damage to Schwann cell and perineural collagen (Mansoor et al. 2020). Corneal sensation and tear break-up time decrease as the duration of diabetes and the HbA1c level increases (Di Zazzo et al. 2021). Neurotrophic keratopathy thus develops because of impaired sensory and trophic function. Corneal nerve degeneration is highly related to the severity of diabetic polyneuropathy (Petropoulos et al. 2013). Even in the prediabetic or early diabetic stage, small peripheral nerve degeneration may exist (Chao et al. 2020). Corneal nerve regeneration can be observed after glycemic control with decreased HbA1c in poorly controlled T2D (Ponirakis et al. 2020, 2021).

Renal Biomarkers Versus Diabetic Keratopathy

The onset of diabetic keratopathy is usually prior to the development of microalbuminuria (Petropoulos et al. 2015). A cross-sectional matched comparison study

showed that a low eGFR level was associated with reduced corneal nerve fiber density, length, and fractal dimension, and UACR was negatively correlated with corneal nerve fiber density and fractal dimension (Tummanapalli et al. 2020). Hyperkalemia is another common complication noticed in chronic kidney disease and has been reported to play a role in the pathogenesis of diabetic polyneuropathy (Arnold et al. 2014). A 24-month prospective, single-blind, randomized controlled trial indicated that controlling serum potassium to a level below 4.5 mEq/L through potassium restriction and medication could slow down the progression of peripheral neuropathy (Arnold et al. 2017).

Effects of Renal Replacement Therapy or Kidney Transplantation on Diabetic Keratopathy

After hemodialysis, central corneal thickness decreases due to removal of excessive body fluid with increase in plasma osmotic pressure. However, both tear break-up time and basal tear secretion measured by Schirmer's test are also decreased, which makes the keratoepitheliopathy score increase (Jung et al. 2013). As for patients of T1D with renal failure, simultaneous pancreas and kidney transplantation can restore normoglycemia and increase eGFR postoperatively, and the operation is beneficial in recovering diabetic polyneuropathy as well. Corneal nerve regeneration with increased corneal nerve fractal dimension also lasts for 6 months after the surgery (Azmi et al. 2019).

Glaucoma in Diabetes

High oxidative stress and vascular autoregulation due to endothelial cell injury in hyperglycemic state will cause retinal ganglion cell apoptosis in diabetic patients (Wong et al. 2011). The formation of advanced glycation end products induces the extracellular matrix remodeling, which can lead to intraocular pressure (IOP) increase because of trabecular meshwork dysfunction (Wong et al. 2011). Despite these predisposing factors for glaucoma in diabetes, the relationship between diabetes and IOP increase is controversial in early studies (Oshitari et al. 2007; Bankes 1967). However, a recent retrospective study including 2524 diabetes patients and 6112 participants without diabetes showed that the IOP was significantly higher in the diabetic group than in the nondiabetic group (Luo et al. 2018). The relationship between HbA1c and glaucoma is contentious as well. Luo et al. found that a higher HbA1c level was significantly associated with a higher IOP after adjusting for the same potential confounders (Luo et al. 2018), while Johnson et al. disclosed that there was no significant association between diabetes control measured by HbA1c and the rate of visual loss or retinal nerve fiber loss in the patients with glaucoma (Johnson et al. 2021).

Renal Biomarkers Versus Glaucoma in Diabetes

Patients with chronic kidney disease also bear a higher risk of developing glaucoma, regardless of the presence of diabetes (Cho et al. 2021). A cross-sectional study showed that decrease in eGFR was associated with IOP elevation, and increase in serum creatinine was associated with increased cupping/disc ratio (Djordjevic-Jocic et al. 2014). A Korean study supported the finding that low eGFR levels were correlated with primary open-angle glaucoma (POAG), while there was no relationship between proteinuria and POAG (Shim et al. 2016). However, another study of East Asian population disclosed that decreased eGFR was not significantly correlated with POAG in the general population (Tham et al. 2020).

Effects of Renal Replacement Therapy or Kidney Transplantation on Glaucoma

A retrospective study in Taiwan disclosed that patients on dialysis had a higher risk of glaucoma, especially angle closure glaucoma (Lim et al. 2020). In the aspect of pathophysiology, hemodialysis will cause significant decrease in plasma osmolarity or relative increase in intracellular urea compared with the extracellular one, and both of them can drive the extracellular fluid to anterior chamber. These will lower the mean arterial pressure and ocular perfusion pressure, and elevate the IOP (Hu et al. 2013). In fact, the amplitude of IOP surge is also related to the speed of plasma osmolarity decrease. Sitprija et al. demonstrated that a rise in IOP would occur with a plasma osmolarity change rate of −11 mOsm/L/h, but not with a rate of −8 mOsm/L/h (SITPRIJA et al. 1964). Other groups found no significant change in IOP with slower rate of plasma osmolarity change (Gafter et al. 1985; Tokuyama et al. 1998; Samsudin et al. 2010). A meta-analysis study indicated that IOP increase during hemodialysis was related to the use of acetate dialysate, which was less frequently observed after replacing acetate dialysate with bicarbonate dialysate in the recent years (Chen et al. 2021). On the other hand, kidney transplantation has a protective effect against glaucoma. A study using Korea National Health Insurance Service Database showed that kidney transplantation could reduce the risk of primary angle closure glaucoma in patients with end-stage renal disease (Moon et al. 2021).

Renal Biomarkers Versus Cataract in Diabetes

For diabetic patients, reduction of glucose to sorbitol catalyzed by aldose reductase through a polyol pathway depicts the main pathophysiology of diabetic cataract. The intracellular accumulation of sorbitol results in hydropic lenses and apoptosis of lens epithelial cell. In addition, high glucose level in aqueous humor induces glycation of

lens proteins, leading to the generation of advance glycation end products and increased oxidative stress (Pollreisz and Schmidt-Erfurth 2010). A case-control study disclosed that victims of diabetic cataract had higher serum sodium, potassium, and chloride levels compared to cataract patients without diabetes, indicating that the rise of electrolytes may cause early cataract formation (Adiga and Harris 2017). Another case-control study not only supported the results of the previous study, but also revealed that serum magnesium level rose in patients with diabetic cataract as well (Kaliaperumal et al. 2021). A cross-sectional study revealed that low eGFR was associated with more severe cataract formation in patients with T2D (Tomić et al. 2021).

Effects of Renal Replacement Therapy or Kidney Transplantation on Cataract

For patients with end-stage renal diseases with or without diabetes, cataract is associated with hemodialysis (Kianersi et al. 2019). The beginning of hemodialysis increased the incidence of cataract surgery (Rim et al. 2016). As for recipients of kidney transplantation, cataract is also a common complication (Ślizień et al. 2020; Raczyńska et al. 2018). This may result from the usage of steroid pulse therapy after transplantation. However, a prospective study evaluating the lens optical density before the operation, 1 month after the operation, and 12 months after the operation showed that the lens opacity decreased in some area at 1 month after kidney transplantation, but then the opacity returned to the level before operation at 12 months. This indicates that kidney transplantation may be able to reverse the diabetic cataract due to better glycemic control, but the usage of steroid may further worsen the lens opacity (Öncül et al. 2021).

Conclusions

Although DN and DR are usually comorbid, different renal biomarkers might have different impacts on diabetes-related ocular diseases and their treatments. Control of DN usually results in improvement or stabilization of DR, but the treatment-associated side effects such as body fluid fluctuation or systemic steroid use might have adverse effects on various diabetic eye diseases.

Applications of Renal Biomarkers to Prognosis

The renal biomarkers can be prognostic for anatomical and probably functional outcomes after initiation of intravitreal injections for diabetic macular edema. Among the various renal biomarkers, severity of albuminuria has a more direct impact than the eGFR on the improvement of central subfield thickness after treatment for retinal edema. Previous clinical data has suggested that severe

albuminuria is linked to more frequent intravitreal injections as a result of less responsive and more severe edema (Liu et al. 2019). The correlations between renal biomarkers and visual prognosis seem to be more complex. In addition to macular thickness, macular ischemia usually predicts suboptimal visual improvement after treatment. Low eGFR in chronic kidney disease has been demonstrated as a biomarker for large non-perfusion areas and lower vascular density in the posterior pole (Min et al. 2020; Vadalà et al. 2019). While there have been reports as for resolution of edema after initiation of hemodialysis or peritoneal dialysis (Takamura et al. 2020; Theodossiadis et al. 2012; Ong et al. 2017), whether improvement of these renal biomarkers can lead to better long-term visual prognosis warrants further investigations.

Mini-dictionary of Terms

- **Albuminuria**: The abnormal presence of albumin in the urine. According to urine albumin-to-creatinine ratio (UACR), albuminuria can be classified into normal to mildly increased (<30 mg/g), moderately increased (30–300 mg/g), and severely increased (>300 mg/g) albuminuria.
- **Diabetic keratopathy**: A degenerative corneal disease seen in patients with diabetes that mainly causes corneal epithelial disturbances.
- **Diabetic macular edema**: A form of diabetic retinopathy that manifests as macula thickening. It is the main reason for blindness in patients with DR.
- **Diabetic retinopathy**: A microvascular complication of diabetes affecting the retina, resulting in the damage of retinal vessels, ischemic change of retina, neovascularization, and loss of vision.
- **Estimated glomerular filtration rate (eGFR)**: An estimation of kidney function using serum creatinine level, age, gender, and body size without the need for 24-h urine collection.

Key Facts of Diabetic Retinopathy

- Diabetic retinopathy (DR) is one of the main microvascular complications of diabetes.
- DR is the leading cause of blindness among working-age adults in developed countries.
- Fundus manifestations include retinal hemorrhage, hard exudate, microaneurysm, macular edema, and neovascularization.
- DR can be classified as non-proliferative or proliferative DR, with the latter involved the development of neovascularization.
- The incidence of DR increased with the duration of diabetes, where 50% of type-1 diabetes patients developed DR 10 years after initial diagnosis.
- Control of blood sugar with HbA1c <7% can slow down the development and progression of DR.

Key Facts of Diabetic Macular Edema

- Diabetic macular edema (DME) accounts for the majority of blindness in DR.
- DME can occur in any stage of DR.
- DME is characterized by macular thickening in various patterns, including diffuse retinal thickening, intraretinal cyst, and subretinal fluid.
- Pathogenesis of DME involves the destruction and hyperpermeability of blood–retinal barrier, where vascular endothelial growth factor (VEGF) plays a crucial role.
- Intravitreal injection of anti-VEGF agents has replaced macular laser photocoagulation and become the mainstay of treatment.

Summary Points

- Renal biomarkers including serum creatinine, eGFR, and UACR are all associated with DR and its severity. Treatment for DN with better control of renal profile is associated with less progression of DR.
- DME patients with albuminuria or low serum albumin tend to have more severe serous-type macular edema at baseline. However, they respond well to anti-VEGF treatment, although more intensive injections might be needed after the loading phase due to disease recurrence.
- Body fluid status is also a biomarker for DME. Overhydration predisposes to the development of macular edema, and hemodialysis is helpful for controlling macular edema in patients with end-stage renal diseases.
- A low eGFR level is associated with poor visual outcome after anti-VEGF treatment for DME. Such association might be attributed to poor macular perfusion in diabetic patients with a low eGFR level.
- The dehydration effect of hemodialysis might enhance the severity of diabetic keratopathy, and the fluctuation of body fluid and plasma osmolarity during hemodialysis might increase the risk of glaucoma.
- Renal transplant may be helpful for DR, DME, diabetic keratopathy, and glaucoma. However, the accompanying use of steroid might result in cataract formation.

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Serum Paraoxonase 1 as a Biomarker: Features and Applications in Type 2 Diabetes Mellitus

20

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Contents

Introduction 436
Structure of PON1 438
Functions and Physiological Roles of PON1 439
PON1 Polymorphisms 440
PON1 Polymorphisms in T2DM 441
PON1 and T2DM 442
Conclusions 443
Application in Other Diseases or Conditions 443
Mini-Dictionary of Terms 444
Key Facts 444
Summary Points 444
References 444

Abstract

Type 2 diabetes mellitus (T2DM) is a complex metabolic and endocrine disorder. Many factors are responsible for the formation and complications of T2DM. One of them is oxidative stress (OS). Recent studies show that paraoxonase (PON)1 activity, an antioxidant enzyme, plays a role in the onset and clinical progression of T2D. In addition to antioxidant properties of PON1, cardioprotective properties have also been demonstrated. These effects have been explained through many cellular mechanisms. At the same time, changes in enzyme activity as a result of PON1 polymorphism have been associated with the development and progression of T2DM. The decrease in PON1 activity has been shown not only in T2DM but also in many diseases such as Alzheimer's and obesity. Both molecular mechanisms and clinical evidence suggest a strong association between PON1 and T2DM.

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Keywords

Type 2 diabetes mellitus · Paraoxonase · Oxidative stress · Polymorphism · High-density lipoprotein · Low-density lipoprotein

Abbreviations

ApoA-I	Apolipoprotein A-I
ApoJ	Apolipoprotein J
Ca	Calcium
CAD	Coronary artery disease
GLUT4	Glucose transporter 4
HbA1c	Glycosylated hemoglobin
HCTL	Homocysteine thiolactone
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model of assessment-insulin resistance
HTLase	Homocysteine thiolactonase
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
LDL	Low-density lipoprotein
MCP-1	Monocyte chemotactic protein-1
MUFA	Monounsaturated fatty acids
OS	Oxidative stress
ox-LDL	Oxidized-LDL
PON	Paraoxonase
PUFA	Polyunsaturated fatty acids
SNPs	Single nucleotide polymorphisms
T2DM	Type 2 Diabetes Mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a condition that occurs as a result of insufficient β-cell insulin secretion in the context of insulin resistance (IR). It is thought that the defect in insulin secretion is influenced by many factors, such as genetics, inflammation, and metabolic stress. However, although the process leading to β-cell dysfunction and death in T2DM has not yet been well defined (American Diabetes Association 2021), oxidative stress (OS) is thought to be an important factor (Galicia-Garcia et al. 2020). In clinical and experimental studies of T2DM, it has been shown that prooxidants and OS-induced tissue damage biomarkers increase, while enzymatic and nonenzymatic antioxidant levels decrease (Rehman and Akash 2017). It is also thought that OS may cause IR by suppressing insulin secretion from pancreatic β cells and reducing glucose uptake in muscle and adipose tissue (McEvoy et al. 2015; Rudich et al. 1998). The role of oxidative stress in the development mechanism of T2DM is summarized in Fig. 1.

The paraoxonase (*PON*) gene family consists of three members, *PON1*, *PON2*, and *PON3*, and all three are located on the long arm of human chromosome 7 (Mahrooz et al. 2018). The amino acid sequences of PONs enzymes are very similar to each other. In addition, the types of substrates in which all three enzymes show enzymatic activity in a single active site are different. PON1 exhibits lactonase, thiolactonase, aryldialkylphosphatase, arylesterase, and paraoxonase activity, while PON2 exhibits lactonase and very low arylesterase activity. PON3 has high lactonase and weak arylesterase activity, but almost no paraoxonase activity (Fig. 2). In addition, although some substrates hydrolyzed by all three PONs are common, the specificity of each enzyme to the substrates may be different. Some substrates of PON1 are phenyl acetate, paraoxon, dihydrocoumarin, and homocysteine thiolactone (Taler-Verčič et al. 2020). While PON1 and PON3 are bound to high-density lipoprotein (HDL) in the circulation, PON2 is located intracellularly.

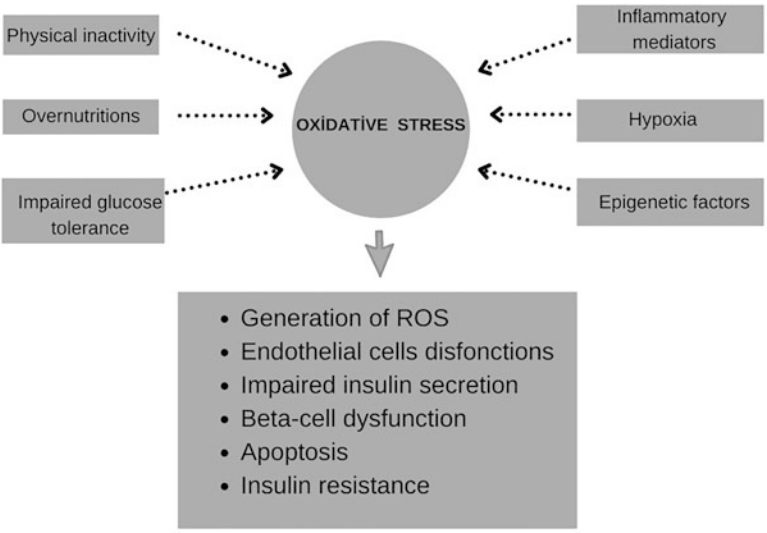

Fig. 1 The role of oxidative stress in the development mechanism of T2DM

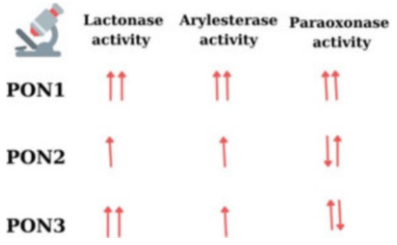

Fig. 2 Enzymatic activity of PON1, PON2, PON3.
Notes: ↑↑:high activity; ↑:low activity; ↑↓:no activity

All three members of this family have antioxidant properties (Mahrooz et al. 2018). PON1 is a glycoprotein with antioxidant and anti-inflammatory effects. Released into the circulation after synthesis, PON1 binds to cell membranes in many tissues and protects them against lipid peroxidation (Deakin et al. 2002a). It also shows antioxidant and atheroprotective effects by preventing low-density lipoprotein (LDL) oxidation of PON1 (Rosenblat et al. 2006).

In T2DM it has been shown that PON1 levels are significantly reduced (Crow et al. 2018; Moya and Máñez 2018). Moreover, it has been found that PON1 activity is inversely proportional to the duration of the disease and metabolic parameters such as plasma glucose concentration, glycosylated hemoglobin (HbA1c) concentration, and homeostatic model of assessment-insulin resistance (HOMA-IR) values, and it has been suggested that the decrease in PON1 activity may contribute to the progression of the disease (Gasecka et al. 2020; Karakaya et al. 2018). The relationship between T2DM and PON1 has been studied through many mechanisms, since PON1 interacts with many cellular pathways. In addition, since similar mechanisms are also effective in the development of diabetes complications, one of the biomarkers examined in the early diagnosis and/or follow-up of these complications is PON1 (Gasecka et al. 2020). At the same time, it has been evaluated that some polymorphisms of *PON1* may be an important factor contributing to the risk of T2DM development (Shokri et al. 2020).

In this chapter, we briefly summarized the structure of PON1, its physiological role, and the association between *PON1* polymorphism and T2DM.

Structure of PON1

PON1 is a protein with a molecular weight of approximately 43 KDa and containing 354 amino acids. It is synthesized in the kidneys and intestines, and especially in the liver (Furlong et al. 2016). PON1 is a calcium (Ca)-dependent hydrolase and is involved in the hydrolysis of a number of organophosphorus insecticides such as parathion, diazinon, and chlorpyrifos, as well as various substrates such as aromatic esters, aromatic and aliphatic lactones, and carbonates (Draganov et al. 2005). PON1 is a six-bladed β-propeller with a central tunnel containing two calcium ions approximately 7.4 Å apart. One of these Ca ions is structural, is located deeper in the central tunnel, and is important for the conformational stability of PON1. The other Ca ion has a catalytic role and is located below the active site gap and takes part in substrate positioning and ester bond activation (Blaha-Nelson et al. 2017).

There are three helices (H1, H2, H3) located in the active site of PON1 and H1 and H2 have a critical role in the interactions of PON1 with the membrane-like surface of HDL (Shunmoogam et al. 2018). PON1-HDL is associated with apolipoprotein A-I (ApoA-I) and apolipoprotein J (ApoJ) (Meneses et al. 2019). ApoA-I is a lipoprotein responsible for the stability of the PON1-HDL interaction (Sorenson et al. 1999) while ApoJ acts as a cytoprotective extracellular chaperone for antioxidant enzymes, including PON1 (Seo et al. 2018; Meneses et al. 2019).

Functions and Physiological Roles of PON1

Antioxidant enzymes play a key role in striking an oxidant–antioxidant balance. PON1 is a protein with antioxidant properties. It is thought to show this effect in two ways. The first of these is via a cytoprotective effect, by hydrolyzing phospholipid hydroperoxides. Another effect is thought to be through the prevention of oxidative change of LDL (Levy et al. 2007). Even if the catalytic Ca ion is removed from the structure of PON1, which is a Ca-dependent hydrolase, its effect on LDL does not change. This suggests that there may be different active sites in PON1 that protect LDL from oxidation (Aviram et al. 1998).

It has been shown that PON1 contributes to the regulation of glucose metabolism by affecting fasting blood glucose levels and insulin sensitivity, and upregulated the expression of glucose transporter 4 (GLUT4) in muscle. PON1 is thought to regulate the expression and translocation of GLUT4 through inhibition of p38MAPK activity. As a result, this leads to decreased insulin receptor substrate-1 (IRS-1) serine phosphorylation and increased IRS-1 tyrosine phosphorylation (Koren-Gluzer et al. 2013) (Fig. 3). In addition, it was determined that PON1 regulated glycolysis, especially through 3-phosphoglycerate, phosphoenolpyruvate, and lactate (García-Heredia et al. 2013).

The effects of PON1 on lipid metabolism have also been reported. In PON1-deficient mice it has been reported that there was a decrease in polyunsaturated fatty acids (PUFA) decreased and an increase in monounsaturated fatty acids (MUFA) increased resulting in a decreased PUFA/MUFA ratio decreased together with a decrease in glycerol and 3-hydroxybutyrate. In addition, a decrease in carnitine levels, which is necessary for the transport of long-chain fatty acids to the mitochondria, was also detected. This change in carnitine levels has been associated with suppression of fatty acid oxidation (García-Heredia et al. 2013).

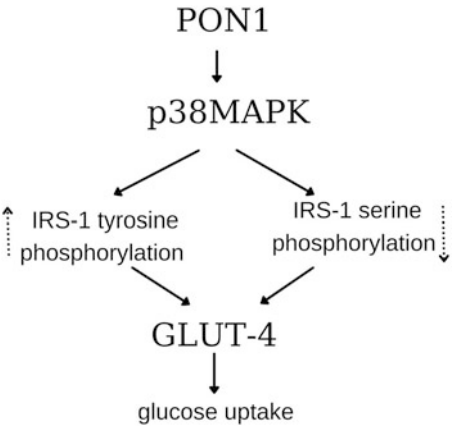

Fig. 3 Effect of PON1 on regulation of glucose metabolism

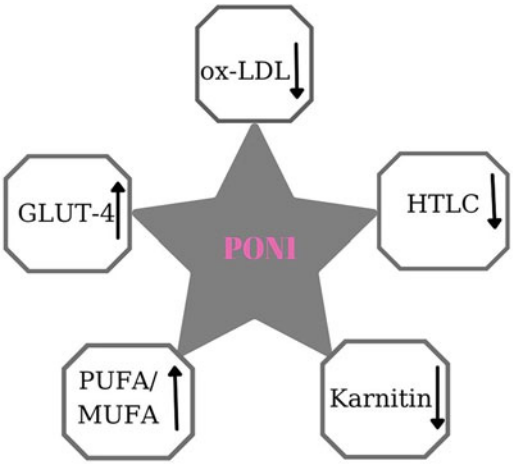

Fig. 4 Physiological effects of PON1

In addition, PON1 hydrolyzes homocysteine thiolactone (HCTL). HCTL is a toxic metabolite formed by the enzymatic conversion of homocysteine in all cell types. It can interact with lysines to alter protein structures, leading to protein inactivation and dysfunction. Increased HCTL levels have been associated with many diseases, including T2DM (Taler-Verčič et al. 2020; Gu et al. 2008). The physiological effects of PON1 are summarized in Fig. 4.

PON1 Polymorphisms

The *PON1* gene is a polymorphic gene with more than 400 single nucleotide polymorphisms (SNPs) identified (Mahrooz et al. 2019). Shokri et al. reported that these polymorphisms affect the enzyme phenotype, and approximately 60% of the changes in enzyme concentration and specific activity are due to genetic factors which include these polymorphisms (Shokri et al. 2020). Four functional SNPs known to affect PON1 levels have been identified in the *PON1* gene. Two of these are in the coding region (at Q192R and L55M), and two are in the promoter sequence(−162 A/G and −108 C/T) (Brophy et al. 2001). It has been reported that the −108 C/T polymorphism is responsible for approximately 12% of the changes in enzyme activity, while the Q192R, L55M and −162 A/G polymorphisms only account for approximately 3% of the effect (Kim et al. 2012) (Fig. 5). The Q192R polymorphism results from the substitution of glutamine at codon 192 with arginine. Allozymes resulting from this polymorphism have been shown to have different protective effects on LDL oxidation (Shokri et al. 2020). The L55M polymorphism is the result of substitution of leucine with methionine at codon 55. This polymorphism has been reported to cause changes in the structure of PON1 (Chiu et al. 2004).

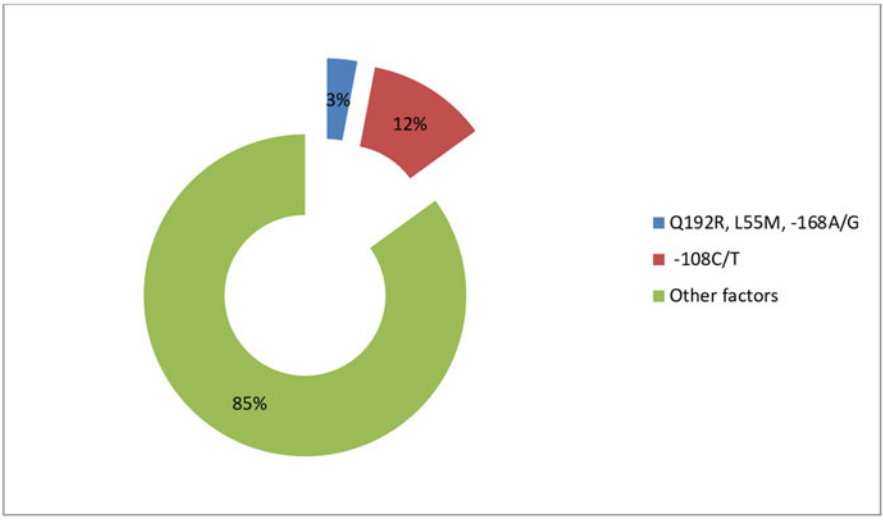

Fig. 5 Effect of single nucleotide polymorphism on the activity of PON1. The −108 C/T polymorphism is responsible for approximately 12% of the changes in PON1 enzyme activity, and the Q192R, L55M, and −162 A/G polymorphisms are responsible for approximately 3%

PON1 Polymorphisms in T2DM

The relationship between PON1 polymorphisms and T2DM and its complications is not yet clear. Gomathi et al. suggested that the Q192R and L55M polymorphisms of PON1 may affect insulin resistance by reducing paraoxonase-1 concentration and regulating GLUT-4 expression (Gomathi et al. 2018). Another study found that the 192 RR and 55 LL genotypes of PON1 were associated with higher PON1 activity than the QQ and MM genotypes, and thus may be more protective against lipid peroxidation. In addition, it was stated that higher prevalence of QQ and MM genotypes in diabetes may be associated with poorer glucose control and therefore advanced nonenzymatic glycation and more oxidative stress (Flekac et al. 2008). Zargari et al. reported that the PON1-Q192R variant had a positive effect on antioxidant capacity in T2DM (Zargari et al. 2016). Deakin et al., in their study on the role of PON1 Q192R and L55M polymorphisms in glucose metabolism, showed that the L55M polymorphism had an independent relationship with the results of the glucose tolerance test. They reported that the L55M polymorphism could affect glucose uptake, thus the MM genotype expressed increased insulin secretion compared to the LL genotype (Deakin et al. 2002b).

In contrast, it has also been suggested that the relationship between PON1 activity and T2DM may be independent of the Q192R, L55M, and −108 C/T polymorphism (Inoue et al. 2000). In addition, it should be noted that gender (Mahrooz et al. 2018) and race/ethnic differences (Luo et al. 2018) may also have an effect on the relationship between PON1 polymorphisms and T2DM susceptibility.

PON1 and T2DM

Since PON1 plays a role in the antioxidant system by preventing the oxidation of LDL, as well as its cytoprotective effect by hydrolyzing phospholipid hydroperoxides, changes in circulating PON1 levels and activity have been associated with many diseases associated with oxidative stress (Navab et al. 1997; Rosenblat et al. 2006; Levy et al. 2007; Meneses et al. 2019).

Chronic hyperglycemia can induce reactive oxygen species and increase OS. Lipid oxidation and lipid peroxide formation are induced due to increased OS (Baynes 1991). PON1 is a protein that has the ability to neutralize lipid peroxides and hydrogen peroxide (Moya and Máñez 2018). Many studies have shown that PON1 level and activity decrease in diabetic patients (Crow et al. 2018; Moya and Máñez 2018). This situation can be due to different mechanisms. One mechanism may be that the reductions in HDL seen in patients with diabetes may cause PON1 to dissociate from HDL and affect its stability. A further mechanism may be that the potential increase in oxidized lipids due to OS may contribute to PON1 inactivation in people with diabetes (Rosenblat et al. 2006). It has also been shown that the activity of PON1 decreases as a result of enzymatic and nonenzymatic glycation. This may reduce PON1 activity in patients with T2DM (Yu et al. 2017). Moreover, PON1 polymorphisms may play a role in the risk of developing diabetes (Farbstein and Levy 2010).

It is known that dyslipidemia develops in T2DM. In this disease, the lipid profile changes, triglycerides and LDL-cholesterol plasma levels increase, and HDL-cholesterol levels and antioxidant activity decrease (Soran et al. 2016). PON1, an antioxidant enzyme associated with HDL, is thought to be associated with the antioxidant and antiatherogenic properties of this lipoprotein (Shokri et al. 2020). Studies have shown that PON1 is also effective in the development and prevention of cardiovascular complications of T2DM. It can do this through different mechanisms, such as lowering plasma oxidized-LDL (ox-LDL) levels, decreasing the ability of macrophages to take up ox-LDL, reducing foam cell formation, promoting macrophage cholesterol efflux, or inhibiting monocyte chemotactic protein 1 (MCP-1) synthesis (Mackness and Mackness 2015; Ng et al. 2008; Rozenberg et al. 2008). Another putative mechanism may be that the decrease in PON1 activity seen in vascular complications of diabetes leads to a decrease in homocysteine thiolactonase (HTLase) activity, resulting in increased HCTL levels, and thus contributing to atherosclerosis in patients with diabetes (Kosaka et al. 2005) (Fig. 6).

PON1 is one of the promising early biomarkers for prediction or detection of early diabetes-related neurodegenerative diseases and neurovascular complications, such as diabetic retinopathy, diabetic neuropathy, and stroke (Gasecka et al. 2020). Mackness et al. reported that low serum PON1 activity may be associated with a tendency toward increased lipid peroxidation in patients with T2DM complicated with retinopathy (Mackness et al. 2000). In another study, HDL-PON1 activity was found to be decreased in patients with T2DM who had either new-onset or overt nephropathy. Decreased PON1 activity has been associated with decreased antioxidant capacity of HDL and thus its inability to protect LDL from oxidation (Li and Gu 2009).

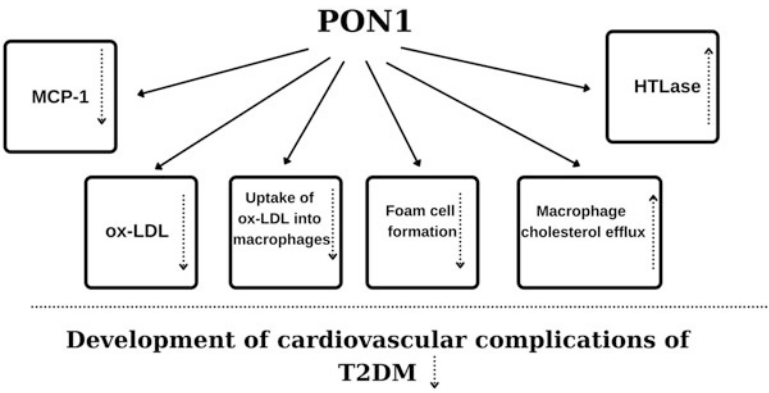

Fig. 6 PON1 can reduce the development of cardiovascular complications of T2DM by many cellular mechanisms

Conclusions

T2DM is one of the most common metabolic disorders worldwide. OS has been implicated as an important factor in the development and progression of T2DM. Decreases in the concentration and activity of antioxidant PON1 may contribute to the development of both T2DM and the complications of T2DM. However, it should be noted that PON1 activity may vary between healthy individuals and also varies in many different diseases. In addition, it should be remembered that there may be a relationship between the changes in PON1 activity caused by PON1 polymorphism and the development of T2DM, and that the activity of PON1 may decrease due to mechanisms such as nonenzymatic glycation in patients with T2DM. However, further work is needed to clarify the mechanisms and functions of PON1 in T2DM.

Application in Other Diseases or Conditions

PON1, an antioxidant effective enzyme, has been associated with many other diseases besides T2DM. It has been associated with atherosclerosis, especially because it causes a decrease in ox-LDL levels and MCP-1 inhibition (Mackness et al. 2004). Another mechanism, associated with coronary artery disease (CAD) and atherosclerosis, is that increased malondialdehyde production due to decreased PON1 activity may adversely affect the function of endothelial HDL in patients with CAD (Annema and von Eckardstein 2016). In addition, another of the atheroprotective effects of PON1 may be that it modulates cholesterol efflux from macrophages (Rosenblat et al. 2006). In studies, a decrease in PON1 activity has been associated with many cancer types, including breast, lung, pancreatic, stomach, colorectal, and ovarian cancer, as well as in Alzheimer's disease, obesity, and non-alcoholic fatty liver disease (Eraldemir et al. 2019; Meneses et al. 2019).

Mini-Dictionary of Terms

- **Glucose transporter 4 (GLUT-4)**: GLUT4 is an insulin-regulated glucose transporter that mediates the transport of glucose to skeletal muscle and adipose tissue.
- **Glycosylated hemoglobin (HbA1c)**: HbA1c is a molecule formed by non-enzymatic binding of hemoglobin and glucose inside the cell, used in the diagnosis of diabetes mellitus.
- **Insulin resistance**: Insulin resistance is a pathological condition in which body cells do not respond properly to the hormone insulin.
- **Paraoxonase**: PON1 is an antioxidant effective protein.
- **Polymorphism**: Polymorphism is a difference in DNA sequence among individuals or groups.
- **Type 2 Diabetes Mellitus**: Type 2 diabetes is a chronic disease characterized by elevated levels of blood glucose.

Key Facts

- Structure of PON1
- Functions and physiological roles of PON1
- PON1 polymorphisms
- PON1 polymorphisms in T2DM
- PON1 and Type 2 Diabetes

Summary Points

- Oxidative stress plays an important role in the development and progression of T2DM and its complications.
- PON1 is an antioxidant and interacts with many cellular pathways.
- Serum PON1 levels are generally observed to be low in T2DM patients.
- Serum PON1 levels are also generally low in complications of T2DM, such as diabetic retinopathy, neuropathy, and nephropathy.
- Although there are still conflicting results, the available evidence to date suggests that the Q192R and L55M polymorphisms of PON1 are associated with T2DM.

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The Ratio of Omega-6/Omega-3 Fatty Acid: Implications and Application as a Marker to Diabetes

21

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Contents

Introduction 451
Fatty Acids 451
General Metabolism of n-3 and n-6 PUFA 453
PUFA Composition and Cell Membrane 454
Role of PUFA in Cellular Function 454
Diet and Polyunsaturated Fatty Acids 456
Omega-6/Omega-3 Ratio 457
Significance of Omega-6/Omega-3 Ratio in Type 2 Diabetes Mellitus 458
Applications to Prognosis, Other Diseases, or Conditions 460
Obesity 460
Cardiovascular Diseases 461
Cancer 461
Pulmonary Disorders 461
Skin 461
Brain-Related Functions 462
Autoimmune Disease 462
Key Facts 462
Summary 463
References 464

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Abstract

Omega-3 and omega-6 groups of polyunsaturated fatty acids (PUFA) are non-interconvertible and metabolically and functionally different, with key opposing metabolic activities in human physiology. The PUFA content of the cell membrane is mostly determined by dietary intake. They are a component of the cellular membrane, improving its fluidity and PUFAs must be released from the membrane by phospholipases in order for signal transmission to occur. Long-chain polyunsaturated fatty acids exert their anti-inflammatory effects by inhibiting lipogenesis and increasing the production of resolvins and protectins. n-3 PUFAs mediate some of these effects by antagonizing n-6 PUFA-induced proinflammatory prostaglandin E formation. Today's industrialized societies with Westernized diet styles have higher overall energy intake, and n-6 PUFAs, but lower energy expenditure. Omega-3 PUFA attenuates ER stress and increases mitochondrial fatty acid β-oxidation and mitochondrial uncoupling. There is competition between omega-3 fatty acids and omega-6 for desaturation enzymes. The unbalanced omega 6/omega 3 ratio in favor of omega 6 PUFAs contributes to the prevalence of atherosclerosis, obesity, and diabetes. n-3 PUFAs are considered to be more protective against inflammation compared with omega 6 PUFA, suggesting the importance of maintaining an ideal balance.

Keywords

PUFA · Omega-3 · Omega-6 · Desaturase · Diet · Diabetes mellitus · Resolvins · Protectins · Maresins · Anti-inflammatory · Arachidonic acid · Alpha-linolenic acid · Linoleic acid · Docosahexaenoic acid · Eicosapentaenoic acid · Docosahexaenoic acid · Prostaglandins

Abbreviations

17-HpDHA	*17*-hydroperoxydocosahexaenoic acid
17S-HDHA	17(S)-hydroxy Docosahexaenoic Acid
5-HEPE	5-Hydroxyeicosapentaenoic acid
ALA	Alpha-linolenic acid
ARA	Arachidonic acid
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
GPR120	G protein-coupled receptor 120
IR	Insulin Resistance
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acids
LTB_4	Leukotriene B4
LTs	Leukotrienes
MaR	Maresins

NEFA	Non-esterified fatty acids
PD	Protectins
PGE_2	Prostaglandin E2
PGs	Prostaglandins
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acids
Rv	Resolvins
SPM	Specialized proresolving mediators
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglycerol
TNF-α	Tumor Necrosis Factor alpha
TXA_2	Thromboxane A2
Δ	Delta
ω	Omega

Introduction

Diabetes mellitus is fast gaining the status of a potential epidemic in India with more than 62 million diabetic individuals currently diagnosed with the disease (Joshi and Parikh 2007; Kumar et al. 2013). In 2000, India (31.7 million) topped the world with the highest number of people with diabetes mellitus followed by China (20.8 million) and the United States (17.7 million) in second and third place, respectively (Kaveeshwar and Cornwall 2014). Insulin resistance (IR) is a key characteristic of type 2 diabetes (T2DM). Improved risk prediction and understanding of the pathogenesis of insulin resistance are needed. Several factors have implication in the etiology of insulin resistance deciphering it as a multifaceted disease.

Lipidomics is a tool that has been used comparatively less in understanding disease pathologies (Shevchenko and Simons 2010). Lipidomics, a subset of metabolomics, provides an understanding of the role of lipids toward the development of obesity and health-related complications, such as Insulin Resistance (IR) and type 2 Diabetes Mellitus (T2DM) (Shevchenko and Simons 2010). This is because circulating lipids reflect an individual's lifestyle (e.g., diet and exercise) as well as their gene and protein activity, all of which can influence IR and T2DM (Schaeffer et al. 2006; Garaulet et al. 2001).

Fatty Acids

Fatty acids (FAs) serve many critical physiological functions including energy reserves, structural components of cell membranes, precursors of eicosanoids, and regulators of gene expression (Nagy and Tiuca 2017; Shetty et al. 2019). Fatty acids influence translocation of glucose transporters and insulin receptor binding and signaling in addition to cell membrane fluidity and permeability. Thus, it is suggestive that FAs may play an essential role in the development of insulin resistance and

type 2 diabetes mellitus. Fatty acids are hydrocarbon chains that have a carboxylic group on one end and a methyl group on the other end.

(A) **Saturated fatty acids (SFAs)**: SFAs do not have double bonds within their carbon backbone. These fatty acids contain 12–22 carbon atoms.
(B) **Unsaturated fatty acids**: Unsaturated fatty acids have one or multiple double bonds. These fatty acids have a chain length of 16–22.

The unsaturated fatty acids are further classified into:

(I) **Mono-unsaturated fatty acids (MUFAs)** having one double bond.
MUFAs can be further divided into *cis* and *trans* subclasses, with the prefix denoting the orientation of the double bond.
(II) **Polyunsaturated fatty acids (PUFAs)** having multiple double bonds. PUFAs can be further categorized into omega 3 (n-3) and omega 6 (n-6) FAs depending on the position of their first double bond from the methylated end. Within these classes, there exist a number of FAs that vary by the length of their carbon backbone and, in the PUFA class, the number of double bonds that they possess.

In the systemic nomenclature of polyunsaturated fatty acids, the Greek letter omega (ω) is used to represent methyl end and delta (Δ) is used to represent carboxylic end. Polyunsaturated fatty acids (PUFAs) are fatty acids with a backbone that contains more than one double bond. PUFAs are classified as omega-3 (n-3) or omega-6 (n-6) based on the position of the terminal double bond in proximity to the molecule's terminal methyl end (Fig. 1). Human beings have the ability to synthesize all fatty acids needed by the body except, linoleic acid (LA, C18:2n-6) and alpha-linolenic acid (ALA, C18:3n-3), (Abbadi et al. 2004). Hence these fatty acids are called essential fatty acids. There comes the significance of providing LA and ALA through the diet. Once we provide it through diet, LA is the precursor for the biosynthesis of n-6 series and ALA is the precursor for the biosynthesis of n-3 series. LA and ALA can be converted to other PUFAs by adding double bonds and acyl chains via desaturases and elongases, respectively. PUFAs with a

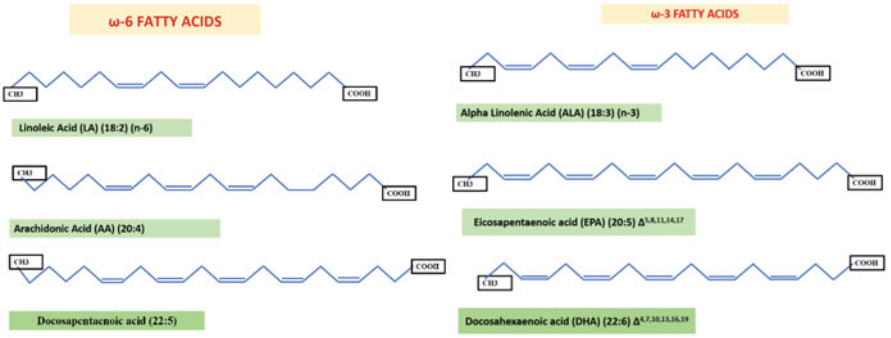

Fig. 1 Structure of w-3 and w-6 PUFA

Table 1 List of common n-3 and n-6 PUFA

Common name	Systematic name	Carbon skeleton and number of double bonds	Carboxyl end reference	Omega family
Linoleic acid (LA)	*all-cis*-9,12-Octadecadienoic acid	18:2	$\Delta^{9,12}$	ω-6
α-linolenic acid (ALA)	*all-cis*-9,12,15-Octadecatrienoic acid	18:3	$\Delta^{9,12,15}$	ω-3
γ – linolenic acid (GLA)	*all-cis*-6,9,12-Octadecatrienoic acid	18:3	$\Delta^{6,9,12}$	ω-6
Eicosatrienoic acid (ETA) (Dihomo-gamma-linolenic acid) DGLA	*all-cis*-8,11,14-Eicosatrienoic acid	20:3	$\Delta^{8,11,14}$	ω-6
Arachidonic acid (AA)	*all-cis*-5,8,11,14-Eicosatetraenoic acid	20:4	$\Delta^{5,8,11,14}$	ω-6
Eicosapentaenoic acid (EPA)	*all-cis*-5,8,11,14,17-Eicosapentaenoic acid	20:5	$\Delta^{5,8,11,14,17}$	ω-3
Docasahexaenoic acid (DHA)	*all-cis*-4,7,10,13,16,19-Docosahexaenoic acid	22:6	$\Delta^{4,7,10,13,16,19}$	ω-3

C=C double bond between the 6th and 7th carbon positions, counting from the terminal methyl end, are referred to as ω-6 PUFAs, while those with the double bond between the 3rd and 4th carbon positions are referred to as ω-3 PUFAs. The letter "n" is also used to indicate where the double bond is located. The physical, metabolic, and physiological properties of PUFAs are greatly influenced by the position of double bonds. According to the systematic nomenclature for PUFAs, indication of the location of double bonds with reference to the first carbon in the carboxylate group is done, for example, when you write 18:2 $\Delta^{9,12}$ for linoleic acid (Table 1); it represents double bonds between carbon 9 and 10, between 12 and 13 carbons.

General Metabolism of n-3 and n-6 PUFA

Linoleic acid and α-linolenic acid are employed as a precursor (Fig. 3), during the production of n-3 and n-6 LC-PUFAs (Di Pasquale 2009; Russo 2009), where they are competitively metabolized in two different routes employing shared enzymes (Sokoła-Wysoczańska et al. 2018). Because they lack the conversion enzymes, n-3-desaturases, mammalian cells, including humans, are unable to convert n-6 to n-3 PUFAs (Ruiz-López et al. 2012). As a result, these two groups are

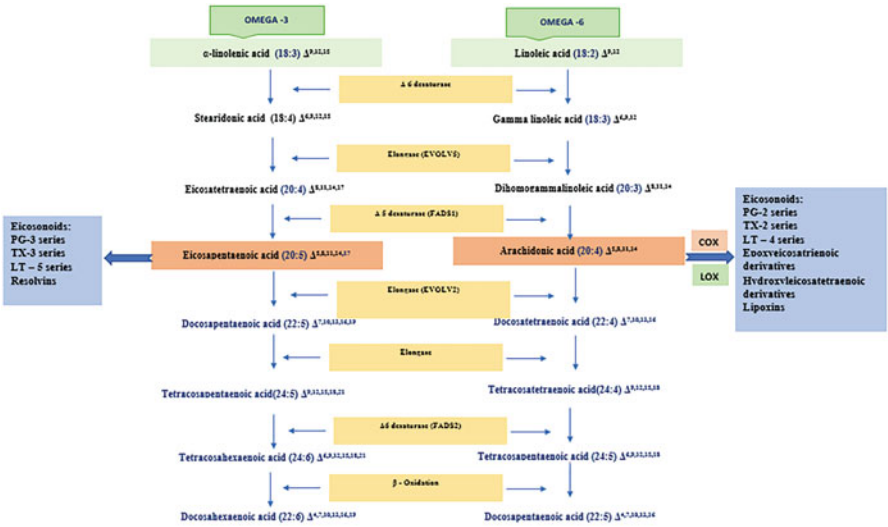

Fig. 2 Elongation and desaturation of Omega-3 and Omega-6 fatty acids

non-interconvertible and metabolically and functionally different, with key opposing metabolic activities in human physiology. Relative plasma levels of individual FAs explain the conversion rates between different FAs and allows estimation of the activity of key enzymes such as the FA desaturases, which seem to play a role in the development of T2DM (Russo 2009) (Fig. 2).

PUFA Composition and Cell Membrane

While the protein makeup of a cell is dictated by genetics, the PUFA content of the cell membrane is mostly determined by dietary intake (Hashimoto and Hossain 2018). Clearly, when humans consume diets that are higher in EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid), the Arachidonic acid (AA) in the cell membranes of almost all cells, including platelets, erythrocytes, neutrophils, monocytes, and liver cells, is partially replaced by these PUFAs (Simopoulos 1994). Inflammation, immunity, blood vessels, and platelets are all controlled in an antagonistic manner by derivatives of n-6 and n-3 PUFAs. In general, n-6 promotes inflammation, platelet aggregation, and vasoconstriction, whereas n-3 promotes vasodilation and inhibits inflammation and platelet aggregation (Bentsen 2017).

Role of PUFA in Cellular Function

In general, n-3 and n-6 PUFAs help the body perform the following three physiologic processes (Burdge 2006) – (1) A rise in β-oxidation. TAG PUFAs modulate transcription factors, which improves fatty acid oxidation, in addition to providing as

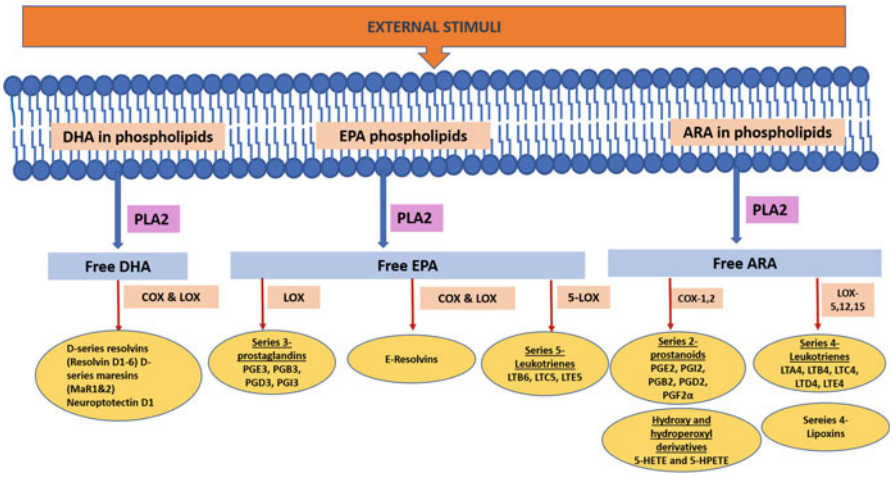

Fig. 3 Role of PUFA in cellular function

a source of fuel. (2) They are a component of the cellular membrane, allowing them to set up and control it, improving its fluidity. Membrane proteins' position, quantity, and function are all optimized by their fluidity. (3) They assist in the transmission of signals. PUFAs must be released from the membrane by phospholipases (Fig. 3) in order for signal transmission to occur. In the cytoplasm, PUFAs influence gene expression either directly or indirectly through lipid mediator substances such as octadecadienoids (C18), eicosanoids (C20), and docosanoids (C22) (Burdge 2006; Balic et al. 2020; Mariamenatu and Abdu 2021).

Previous research has established that long-chain polyunsaturated fatty acids (LC-PUFAs), specifically eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are precursors of signaling molecules (bioactive lipid mediators) and essential components of cell membranes in animals and humans (Casula et al. 2013; Dyall 2015; Weiser et al. 2016; Calder 2018). Eicosanoids, resolvins, and protectins, which are generated from EPA and DHA (Calder 2013), evidently function through receptors. RvD1, RvD2, PDX, RvE1, and 5-HEPE, bioactive lipids generated from EPA or DHA, have recently been shown to influence insulin resistance and pancreatic-cell function. RvD1 reduces tissue inflammation and thereby increases insulin sensitivity (Bathina and Das 2021).

Recent studies also indicate anti-inflammatory and insulin-sensitizing effects of these fatty acids in metabolic disorders. Classically, n-3 PUFAs mediate some of these effects by antagonizing n-6 PUFA (arachidonic acid)-induced proinflammatory prostaglandin E2 (PGE2) formation. The lowering of nuclear factor-B activation is another well-known method by which n-3 PUFAs exert their anti-inflammatory effects. This transcription factor is a strong inducer of proinflammatory cytokine production, such as interleukin 6 and tumor necrosis factor-α (Fig. 4), which are both reduced by EPA and DHA. Other research suggests that n-3 PUFAs reduce inflammation by inhibiting lipogenesis and increasing the production of resolvins and protectins. Although the pathogenesis of type 2 diabetes is related to

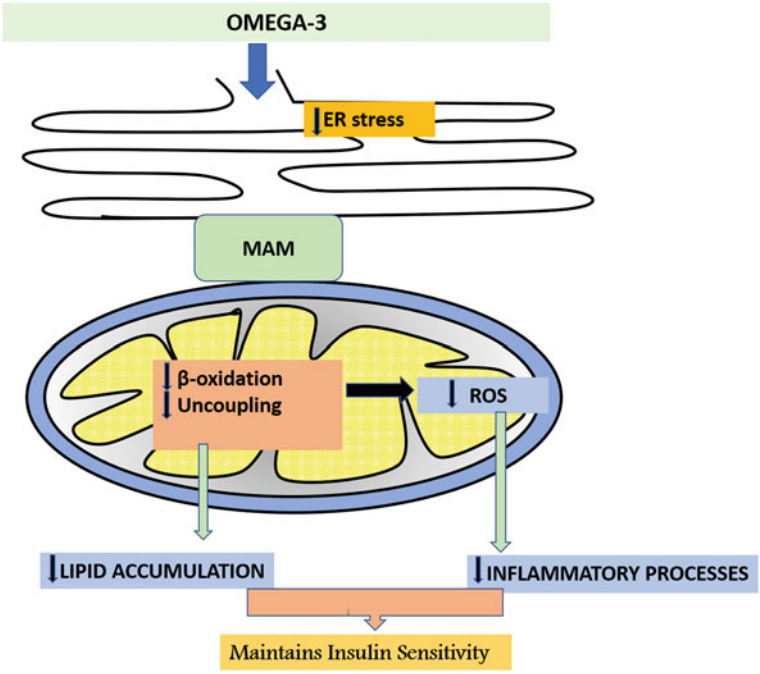

Fig. 4 Omega-3 PUFA effects on insulin sensitivity

pro-inflammatory mechanisms (Yary et al. 2016), DGLA, which is metabolized to the anti-inflammatory eicosanoid, prostaglandin (PG) E1, via the cyclooxygenase (COX) pathway, and pro-inflammatory arachidonic acid, prostaglandin 2- series-thrombaxane A2, leukotrienes B4 was associated positively with type 2 diabetes risk (FBS, HbA1c, insulin, and HOMA-IR) (Kim et al. 2011; Yary et al. 2016; Shetty et al., 2020).

Diet and Polyunsaturated Fatty Acids

According to Simopoulos (2006, 2008), the whole human diet, including energy intake and expenditure, changed agricultural revolution (10,000 years ago). However, considerable changes in the human diet have occurred since the industrial revolution (150–160 years ago), particularly in the kind and amount of both essential and conditionally essential fatty acids. Today's industrialized societies with Westernized diet styles have higher overall energy intake, n-6 PUFAs, but lower energy expenditure, as well as lower intake of n-3 PUFAs, complex carbohydrates and fiber, fruits and vegetables, protein, antioxidants, calcium, and vitamin D (Simopoulos 2011).

Table 2 Evolutionary norms of the n-6 to n-3 PUFA

Year	n-6/n-3 ratio
1935 and 1939 (Chaves et al. 2019)	8.4/1
1985 (Chaves et al. 2019)	10.3/1–12.4/1
2001 and 2011 (Simopoulos 2002, 2004, 2008, 2010, 2011; Molendi-Coste et al. 2011)	15–16.7/1
2016–2018 (Sokoła-Wysoczańska et al. 2018; Simopoulos 2016)	20/1
2019 (Chaves et al. 2019)	50/1

To date, several studies have been extensively done regarding the ratio of n-6 to n-3 and it is thought that our forefathers absorbed n-6 and n-3 in a 1-2/1 ratio during the Paleolithic period (evolutionary times), which is considered to be a perfect and balanced ratio (Simopoulos 2004, 2006, 2008; Bhardwaj et al. 2016). But during the course of human evolution, there has been a gradual change in the evolutionary norms of the n-6 to n-3 PUFA ratio followed by a drastic change in diet pattern due to Industrial Revolution (Table 2).

Overall, these studies indicate that intake of n-6 PUFAs has increased and that of n-3 has decreased, resulting in a large increase in the n-6/n-3 ratio, which is now 20–50 times that of evolutionary times. This shift in the n-6/n-3 ratio is perhaps critical more than any other dietary factor, since it can contribute toward significant physiological changes in the human body. Much of the instability in n-6/n-3 ratio in body tissue may result in systemic inflammation, as well as in overweight/obesity, all of which contribute to an epidemic of diet-related chronic noncommunicable diseases like coronary heart disease, hypertension, cancer, type 2 diabetes, arthritis, and other autoimmune and possibly neurodegenerative diseases (Chaves et al. 2019).

Omega-6/Omega-3 Ratio

Aquatic habitats are considered as the biosphere's primary supply of LC-PUFAs (Pereira et al. 2004) because only specific taxa of algae can efficiently synthesize EPA and DHA from beginning (de novo). The EPA and DHA produced by algae are transmitted to higher trophic level species, such as invertebrates and fish, and eventually to terrestrial consumers, such as humans, via trophic chains. Given the origin of n-3 LC-PUFA in the marine environment, it is no surprise that practically all of the EPA and DHA in our diet come from fish (Gladyshev and Sushchik 2019) and seafood, traditionally from capture fisheries. The evidences presented thus far supports the idea that most animals can only get ALA from food because it is generated by plants (Uttaro 2006; Zhou et al. 2011). It is worth noting that, unlike EPA and DHA, which are produced by algae, ALA is produced by terrestrial vascular plants and is the primary component of chloroplast photosynthetic membranes (Harwood 1996; Sayanova and Napier 2004; Ward and Singh 2005; Ruiz-Lopez et al. 2012).

However, with an ever-increasing global population, demand for fish and seafood has steadily increased, and aquaculture has increasingly filled this demand, with fish and seafood farming. As a result, almost all of the EPA and DHA present in fish come from their diet, natural prey organisms in wild-caught food fish, or fish feed in farmed fish (Tocher 2015). The only method to assure that farmed fish had high levels of EPA and DHA was to add these fatty acids in the feed, which necessitated large amounts of fish oil and fishmeal, the majority of which originated from limited and depleting marine resources (FAO 2016). Modern aquaculture produces fish that contain fewer n-3 PUFAs than fish grown naturally in the wild oceans, rivers, and lakes (Mariamenatu and Abdu 2021).

Significance of Omega-6/Omega-3 Ratio in Type 2 Diabetes Mellitus

The proposed mechanism of how omega 3 improves insulin sensitivity is that omega-3 PUFA attenuates ER stress and increases mitochondrial fatty acid β-oxidation and mitochondrial uncoupling with subsequent decrease in lipid accumulation and ROS production (Fig. 4) (Ertunc and Hotamisligil 2016), thereby improving insulin sensitivity by downregulation of inflammasome/inflammatory processes (Lepretti et al. 2018). Mfn2 is involved in mitochondrial dynamics homeostasis and MAM integrity maintenance, and omega 3 is said to have a positive effect on Mfn2. Omega 3 PUFA have also positive effects on Mfn2 involved in maintenance of mitochondrial dynamics homeostasis and MAM integrity (Lepretti et al. 2018).

EPA and DHA regulate insulin sensitivity (Akt phosphorylation), glucose utilization, in part mediated by PPAR-γ and AMPK activation (Perez-Matute et al. 2007). AMPK is a recognized therapeutic target for T2DM (Wang and Chan 2015) and is the main target activated by metformin. EPA and DHA are involved in the regulation and secretion of adipokines involved in intermediate metabolism, energy homeostasis, and lipid and glucose metabolism. DHA prevents inflammation and lipotoxicity, restoring insulin sensitivity (Mazoochian et al. 2018). Other mechanisms involved in the modulation of insulin secretion from pancreatic β- cells by omega 3 fatty acids are directly by lipid raft structure and function alteration and indirectly by inhibition of the expression of pro-inflammatory mediators in adipose tissue and promotion of adipokine production (Mansouri et al. 2018). Omega-3 PUFAs inhibit inflammatory cytokine and eicosanoids production from AA and induce adipokine production from adipose tissue and directly affects β-cell function by binding to PPARs, GPR40, and GPR120 thereby promoting insulin secretion (Mansouri et al. 2018) (Fig. 5). Clària et al. (2017) found that white adipose tissue is critical for maintaining metabolic and energy balance, also AA, EPA, and DHA all have an impact on this balance. Clària et al. (2017) showed specialized pro-resolving mediator production in white adipose tissues at increased levels of EPA and DHA. The biological mechanisms by which different types of PUFAs might affect glucose metabolism remain to be elucidated (Tsurutani et al. 2018).

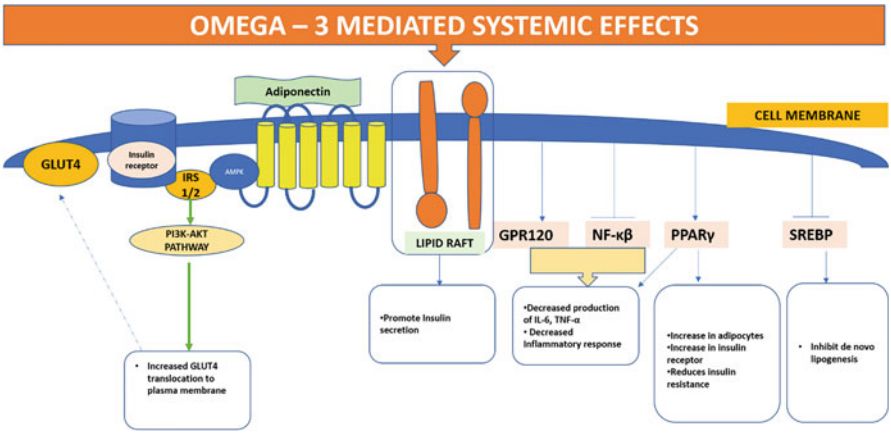

Fig. 5 Omega-3-mediated systemic effects in metabolic pathways

Previously, the association of higher serum total omega-3 PUFA and D5D activity were associated with a lower risk of incident T2DM, and higher GLA and DGLA levels and D6D activity were associated with a higher risk (Das 1994). Studies also have demonstrated that, a defect in the activity of the enzymes D6 and D5 desaturases, key enzymes in PUFA metabolism, is a factor in the development of insulin resistance syndrome (Hainault et al. 1993). Therefore, factors affecting the activity of these desaturases may have public health implications.

Overall, the increase in free fatty acid composition in the body concerning insulin resistance can be deciphered with the insulin binding to its insulin receptor. In normal condition, binding of insulin to its receptor in the adipose tissue cell membrane triggers intracellular signal that suppress the activity of HSL (hormone sensitive lipase), an intracellular enzyme from adipocyte, which hydrolysis lipids such as triglycerides. When an individual is insulin resistant, there is an intracellular signal suppression, thereby increasing HSL activity that hydrolysis TG to glycerol and free fatty acids (FFA), which in turn is released to the circulation in blood and moves toward the liver. These fatty acids are taken up by the liver hepatocytes and are channeled to their secretary pathways. Due to insulin resistance esterification increases. The enzyme LPL (Lipoprotein lipase) in the blood vessels hydrolysis monoglycerides and FFA. This process continues thereby increasing the fatty acid composition (Hainault et al. 1993; Mori et al. 1997; Peyron-Caso et al. 2002; Rimoldi et al. 2001; Mathias et al. 2014; Wu et al. 2014). There is always a competition between omega-3 fatty acids and omega-6 for the desaturation enzymes. ALA is preferred by both fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) to LA (Simopoulos 2009). In a physiological state, a balance between the omega 6 and omega 3 fatty acids is less inflammatory in terms of gene expression (Simopoulos 2015), prostaglandin and leukotriene metabolism, and interleukin-1 (IL-1) production (Simopoulos 2015). The unbalanced omega 6/omega 3 ratio in favor of omega 6 PUFAs is highly prothrombotic and pro-inflammatory, which

contributes to the prevalence of atherosclerosis, obesity, and diabetes (Simopoulos 2015).

The properties of omega-6 and omega-3 fatty acids in the diet are a determinant of biochemical efficiency, which is essential in providing the optimal conditions for human development as described previously, enzymes involved in the metabolism of the LA and ALA are shared. There is competition between them and the omega-6 and omega-3 fatty acids also regulate each other (Day et al. 2017). Increase in omega-3 PUFA results in reduced synthesis of highly pro-inflammatory eicosanoids from AA. PGs (one-series) from DGLA are anti-inflammatory eicosanoids; however, the amount of DGLA in the body is about tenfold lower than that of AA. According to reports, the target ratio of omega 6: omega 3 ratio for health should be 1:1 to 2: (Day et al. 2017). From the study, the omega 6: omega 3 ratio in nondiabetic subjects was 4:1 and in diabetic subjects the ratio was 13:1. Despite the intake of low-fat diet by the Indian population, CAD incidence is high and high omega-6/omega-3 ratios may be the culprit. The Indo-Mediterranean Diet Heart Study findings, as well as other intervention trials and epidemiological studies have indicated that a dietary modification may induce positive changes in the dietary omega-6/omega-3 fat ratio (Day et al. 2017). Thus, n-3 PUFAs are considered to be more protective against inflammation compared with omega 6 PUFAs, suggesting the importance of maintaining an ideal balance.

Applications to Prognosis, Other Diseases, or Conditions

While the human diet has evolved dramatically over the last 10,000 years, our DNA has remained relatively unchanged. It.is not unexpected that newly established Western diets poor in -3 PUFAs and abundant in -6 PUFAs promote the development of many chronic inflammatory disorders, given that humans are naturally accustomed to the food on which they evolved, and their genetic patterns were created. A Western diet with a higher omega 6 intake and omega-6/3 ratio bolsters pathogenesis of many diseases like cancer, cardiovascular disease, inflammatory and autoimmune diseases, whereas lower a low omega-6/omega-3 ratio may revert the adverse effects.

Obesity

Increased obesity in the progeny is linked to high consumption of omega-6 fatty acids during the perinatal period. The level of AA in adipose tissue has been linked to children's BMI and overweight status in human research. High omega-6/omega-3 fatty acids in the membrane phospholipids of umbilical cord red blood cells (RBCs) were linked to high subscapular skin-fold thickness at 3 years of age. Studies on intervention with a combination of -6, gamma-linolenic acid (GLA), and -3 long-chain PUFAs appear to have the most potential for reducing inflammatory processes, which could be helpful for treating inflammatory skin illnesses such as atopic dermatitis, psoriasis, and acne. Along with physical activity, a balanced omega-6/

omega-3 ratio of 1–2/1 is one of the most important dietary factors in preventing obesity. In the treatment of obesity, a lower omega-6/omega-3 ratio should be considered. EPA and DHA supplementation has been proven in animal and human trials to be protective against obesity and to prevent weight gain in obese animals and humans (Donahue et al. 2011). Studies in rats fed with high lipid diets, including omega-3 PUFAs, showed a reduction in visceral (epididymal and/or retroperitoneal), and the impact was dose-dependent (Simopoulos et al. 2016).

Cardiovascular Diseases

Over the last 20 years, fatty acid supplementation has been extensively researched and documented in treating cardiovascular and cerebrovascular disorders such as coronary artery disease (CAD), arrhythmia, and cerebral vascular accident (CVA) (Simopoulos et al. 2016).

Cancer

Given the importance of fatty acids (FAs) in cancer development, finding treatments that target FA metabolic reprogramming has piqued clinical interest. A major chunk of inhibitors developed target enzymes specifically involved in de novo FA synthesis and exogenous lipid uptake, but there is also renewed interest in delineating synergistically how specific dietary interventions may improve the efficacy of existing cancer therapeutics (Koundouros and Poulogiannis 2020).

Pulmonary Disorders

In an asthmatic population, Broughton et al. (1997) investigated the efficacy of omega-3 fatty acids at a 10/1 to 5/1 omega-6/omega-3 ratio in alleviating methacholine-induced respiratory distress. Methacholine-induced respiratory discomfort increased when omega-3 levels were low. In 40% of the test patients (responders), changes in urine 5-series leukotriene excretion indicated treatment success and a dose change, but nonresponders experienced a further reduction in respiratory capacity. Adams et al. (2019) has also demonstrated omega 3 protective against asthma. A 4-series to 5-series of 1 urinary ratio produced by omega-3 fatty acid consumption may predict respiratory improvement (Simopoulos 2016).

Skin

There is an increasing number of promising studies in favor of supplementing with a combination of GLA and -3 LC-PUFAs, which shows the most significant promise in reducing inflammatory processes and improving chronic inflammatory diseases,

including chronic inflammatory skin diseases such as AD, psoriasis, acne, and to a lesser extent hidradenitis suppurativa.

Brain-Related Functions

According to evidence from multiple cross-sectional studies, an increase in omega-6 to omega-3 fatty acids (FAs) may have a deleterious effect on cognition in old age (Andruchow et al. 2017). Studies have also shown that children with epilepsy have aberrant omega-3 to omega-6 serum level ratios, which is linked to cognitive impairment, based on our data. Early detection of cognitive impairment and appropriate intervention are required to improve quality of life and prevent learning difficulties and social problems in these children. According to National Health and nutrition examination Survey (NHANES) 2011–2014, intake of dietary -3 and -6 fatty acids may be inversely related to poor cognitive performance (Dong et al. 2020).

Humans produce proinflammatory cytokines such as interferon gamma (IFNc), TNFa, IL-6, and IL-1 in response to psychological stress. Overproduction of proinflammatory cytokines is caused by an imbalance of omega-6 and omega-3 PUFA in the peripheral circulation. Changes in fatty acid content are thought to play a role in the pathophysiology of major depression (Simopoulos 2016).

Autoimmune Disease

Clinical studies on genetic mouse models have delineated the underlying mechanisms and function of omega fatty acids and their metabolites in prevention and treatment of systemic lupus erythematosus (SLE), type 1 diabetes, rheumatoid arthritis, and multiple sclerosis over the past decade (Li et al. 2019).

Key Facts

- Linoleic acid and α-linolenic acid are employed as a precursor during the production of n-3 and n-6 LC-PUFAs.
- LA and ALA are not interconvertible and compete for the rate-limiting Δ6-desaturase in the synthesis of long-chain PUFA.
- The parent molecules for the formation of eicosanoids are AA (omega-6) and EPA (omega-3). Eicosanoids from AA exhibit characteristics that are fundamentally opposed to those of EPA.
- Increased dietary omega-6 EFA intake alters the physiological state to one that is prothrombotic, proconstrictive, and proinflammatory.
- PUFAs influence gene expression either directly or indirectly through lipid mediator substances such as octadecadienoids (C18), eicosanoids (C20), and docosanoids (C22).

- Today's industrialized societies with Westernized diet styles have higher overall energy intake of n-6 PUFAs, but lower energy expenditure and lower intake of n-3 PUFAs.
- As a result, lowering omega-6 intake and increasing omega-3 intake is critical in the prevention and management of chronic disease. Furthermore, for homeostasis and optimal growth, the ratio of omega-6 to omega-3 fatty acids is critical.

Summary

- Insulin resistance (IR) is a key characteristic of type 2 diabetes (T2DM). Improved risk prediction and understanding of insulin resistance are needed. Lipidomics provides an understanding of the role of lipids toward the development of obesity and health-related complications.
- Fatty acids are hydrocarbon chains that have a carboxylic group on one end and a methyl group on the other end. Polyunsaturated fatty acids (PUFAs) are fatty acids with a backbone that contains more than one double bond. The physical, metabolic, and physiological properties of PUFAs are greatly influenced by the position of double bonds.
- Linoleic acid and α-linolenic acid are employed as a precursor to n-3 and n-6 LC-PUFAs. These two groups of PUFAs are non-interconvertible and metabolically and functionally different, with key opposing metabolic activities in human physiology.
- The PUFA content of the cell membrane is mostly determined by dietary intake. In general, n-6 promotes inflammation, platelet aggregation, and vasoconstriction, whereas n-3 promotes vasodilation and inhibits inflammation.
- PUFAs modulate transcription factors, which improves fatty acid oxidation, in addition to providing as a source of fuel. They are a component of the cellular membrane, allowing them to set up and control it, improving its fluidity. PUFAs must be released from the membrane by phospholipases in order for signal transmission to occur.
- Long-chain polyunsaturated fatty acids (LC-PUFAs) exert their anti-inflammatory effects by inhibiting lipogenesis and increasing the production of resolvins and protectins. N-3 PUFAs mediate some of these effects by antagonizing n-6 PUFA (arachidonic acid)-induced proinflammatory prostaglandin E formation.
- The whole human diet, including energy intake and expenditure, changed agricultural revolution. However, considerable changes in the human diet have occurred since the industrial revolution. Today's industrialized societies with Westernized diet styles have higher overall energy intake, n-6 PUFAs, but lower energy expenditure.
- The ratio of PUFA to PUFA in the human body is 20–50 times that of evolutionary times. This shift in the n-6/n-3 ratio is perhaps critical more than any other dietary factor, since it can contribute toward significant physiological changes in the human body.

- Most animals can only get EPA and DHA from food because it is generated by plants. Aquaculture has filled this demand, with fish and seafood farming. Modern aquaculture produces fish that contain fewer n-3 PUFAs than fish grown naturally in the wild.
- Omega-3 PUFA attenuates ER stress and increases mitochondrial fatty acid β-oxidation and mitochondrial uncoupling, thereby improving insulin sensitivity by downregulation of inflammasome/inflammatory processes. Omega 3 PUFA have also positive effects on Mfn2 involved in homeostasis and MAM integrity.
- EPA and DHA regulate insulin sensitivity, glucose utilization, and lipotoxicity. AMPK is a recognized therapeutic target for T2DM and is the main target activated by metformin. Omega-3 PUFAs inhibit inflammatory cytokine and eicosanoids production from AA and induce adipokine production from adipose tissue.
- There is competition between omega-3 fatty acids and omega-6 for desaturation enzymes. The unbalanced omega 6/omega 3 ratio in favor of omega 6 PUFAs contributes to the prevalence of atherosclerosis, obesity, and diabetes. N-3 PUFAs are considered to be more protective against inflammation compared with omega 6 PUFA, suggesting the importance of maintaining an ideal balance.

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Engulfment and Cell Motility Protein (ELMO)-1 as a Biomarker in Type II Diabetes

22

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Contents

Introduction 470
A Genetic Variant of ELMO-1 as the Risk for DN in T2DM 471
The Role of ELMO-1 in Diabetic Nephropathy, Studies in the Animal Model, and Cell Culture 472
ELMO-1 in Circulation and Renal Tissue in Humans as a Potential Biomarker of DN in T2DM 477
Application to Prognosis, Other Diseases or Condition 478
Mini-Dictionary 480
Key Facts 480
Key Facts of Albuminuria 480
Key Facts of ELMO-1 Protein 480
Key Facts of ELMO-1 Genetic Variant rs741301 481
Summary Points 481
References 481

Abstract

Increasing prevalence of T2DM in parallel with increased prevalence of DN, which leading cause of ESRD and T2DM. Albuminuria as a standard biomarker

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to monitoring the progression of DN know has been challenging due to its ability to detect early renal lesions, monitoring the progression of renal lesion and treatment to prevent ESRD. Discover novel biomarker performed recently to focus on finding ideal marker of a cellular process that can reflect the renal function and histology appearance of DN progression. This chapter presented a review of ELMO-1 as one of the candidates of a biomarker of DN progression, which is reporting in genomic, transcriptomic and metabolomic levels. Previous studies reported genetic variant of ELMO-1 as risk susceptibility for suffering in DN, which replicated in many populations worldwide. In addition, genetically modified animal models and cell lines that have increased ELMO-1 expression reported worsening DN progression. Parallel with animal study, ELMO-1 protein was associated with a marker of declining renal function in T2DM patients.

Keywords

Albuminuria · Biomarker · Diabetic Nephropathy · ELMO-1 · End-Stage Renal Diseases · Extracellular matrix · Genetic Variant · Intron · Stress Oxidative · Type 2 Diabetes Mellitus

Abbreviations

ACR	Albumin creatinine ratio
ARMS-PCR	Amplification-refractory mutation system- polymerase chain reaction
DN	Diabetic nephropathy
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ELMO-1	Engulfment and cell motility protein
ESRD	End-stage renal diseases
GWAS	Genome-wide association study
ILK	Integrin like kinase
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
T2DM	Type 2 diabetes mellitus
TGF-ß	Transforming growth factor- ß
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

Introduction

The prevalence of Type 2 Diabetes Mellitus (T2DM) is increasing worldwide, which cause increasing the prevalence of diabetic nephropathy as the most frequent of microvascular complications. Diabetic nephropathy (DN) is the leading cause of end-stage renal diseases (ESRD) and mortality in T2DM. This cause burden of health cost and patient's quality of life (Koye et al. 2018; Persson and Rossing 2018).

Finding such an ideal biomarker that could monitor disease progression was needed to understand pathophysiology better and enhance treatment.

The ideal biomarker for the diseases should be non-invasive, easy to measure, inexpensive, readily available sources, high sensitivity to detect early renal lesions of DN, high specificity, monitor the treatment, risk stratification, and possess prognostic value and biologically plausible. The biomarker for DN used widely does not yet meet those ideal criteria for biomarker (Biomarkers Definitions Working Group 2001; Bennett and Devarajan 2017).

Albuminuria has been using widely for monitoring DN progression. Recently, the interpretation of this marker is challenging. The previous study reported that microalbuminuria could regress to normo-albuminuria. Patients who have declined glomerular filtration rate < 60 ml/minute/m^2 was not suffering microalbuminuria. In addition, patients with normo-albuminuria may have a histopathology appearance that reflected nephropathy (Currie et al. 2014; MacIsaac et al. 2014; Persson and Rossing 2018). Histopathology appearance of DN was glomerular-based membrane thickening, epithelial to mesenchymal transition, a vascular injury which leads to glomerular sclerosis that describes the best for DN progression. The cellular process occurred before and continued in parallel with decline renal function. Unfortunately, it could detect from renal biopsy, a relatively invasive procedure that needs enormous resources to be performed (Roshan and Stanton 2013; Gonzalez Suarez et al. 2013; Espinel et al. 2015). The discovery of an ideal biomarker for DN recently focus on the marker of a cellular process associated with renal function and histology appearance, which promise a potency as an ideal biomarker of DN (Batlle 2003; Currie et al. 2014; Uwaezuoke 2017; Lee and Lam 2015).

Engulfment and cell motility protein (ELMO)-1 was known before as a protein that had played a role in apoptosis and cell migration. Then Genome-wide association study (GWAS) tagging that the genetic variant of the ELMO-1 gene is associated with DN in Japanese (Shimazaki et al. 2005). That phenotype and genotype association study replicate in many other populations worldwide and potentiate as a genetic risk associated DN (Leak et al. 2009; Mehrabzadeh et al. 2016; Mooyaart et al. 2011; Omar et al. 2021; Pezzolesi et al. 2009; Wu et al. 2013). Furthermore, the role of ELMO-1 protein in DN progression has been investigating in various animal models and cell cultures. This protein plays a role in depositing extracellular matrix, cell apoptosis, increasing oxidative stress (Hathaway et al. 2016; Sharma et al. 2016; Shimazaki et al. 2006). A study in humans reported the correlation of ELMO-1 with a clinical marker of renal function in T2DM (Elfiani et al. 2020). It showed the potential of ELMO-1 as a DN biomarker in T2DM patients. This chapter will review the potency of ELMO-1 as a biomarker of DN in T2DM.

A Genetic Variant of ELMO-1 as the Risk for DN in T2DM

The genetic variant is an unmodified risk factor for the diseases, but the screening can identify the risk for diseases or predict the prognosis for diseases (Baptista 2005; Frezzo et al. 2003). Meta-analysis and review studies reported the ELMO-1 genetic

variant as a risk factor for diabetic nephropathy in diabetes patients (Mooyaart et al. 2011; Wei et al. 2018).

A genetic variant of ELMO-1 was first studied associated with DN in T2DM in the Japanese population. The genetic variant in intron 18 + 9170 A/G (rs741301) was the strongest associated with DN in T2DM. Patients with GG genotype are more susceptible (OR: 95%CI; 2.67:1.71-4.16) for suffering DN (Shimazaki et al. 2005). These results are replicating by studies performed in Chinese, Iranian, South Indian, and Egyptian (Bodhini et al. 2016; Omar et al. 2021; Hou et al. 2019; Mehrabzadeh et al. 2016; Wu et al. 2013). A study in Jingzou Chinese reported the different risk allele. The patients who have the A allele are more susceptible to suffering DN (Table 1) (Wu et al. 2013).

The others studies in North Indian, Egyptian, and Tunisian Arab reported this genetic variant rs741301 as the risk for T2DM but not DN in T2DM. The A allele is more susceptible to suffering T2DM than the G allele in South India (Turki et al. 2018; Omar et al. 2021; Yadav et al. 2014). In addition, a study in Malaysia and Mexican Americans reported this genetic variant not associated with the risk of DN in T2DM patients (Table 1) (Kim et al. 2010.; Yahya et al. 2019).

Another genetic variant associated with DN in T2DM located in the intron 13, intron 20 and 3′ flanking region was DN genetic risk factor (Table 2). The genetic variants rs1345365 and rs10951509 located in the intron 13 are associated with DN in T2DM African American, American Indian population, Chinese Han, which has minor allele as a protective factor (Hanson et al. 2010; Hou et al. 2019; Leak et al. 2009). In addition, genetic variant rs10255208 located in the 3′ flanking region and rs7782979 located in intron 20 associated with DN in the Chinese Han population (Hou et al. 2019).

The functional study of this intronic genetic variant is not yet elucidated. However, the intron is not a coding region, but it induced an aberrant splicing site. Which could cause exon skipping, cryptic splice site utilization for enhancers, and silencers that influence the stability and level of protein that encode (Pagani and Baralle 2004; Cooper 2010). In addition, it could change modulator protein binding. Shimazaki et al. presumed GCR-1 protein is compatible with the sequences of rs741301. This protein is a modulator protein induced by high glucose concentration. This protein binding to sequences of this genetic variant could control the transcriptional activity of the glycolytic enzyme in response to the alteration of extracellular glucose concentration. It may explain the function of this genetic variant as a risk factor for DN (Shimazaki et al. 2005).

The Role of ELMO-1 in Diabetic Nephropathy, Studies in the Animal Model, and Cell Culture

The ELMO-1 protein is known for its role in engulfing apoptotic cells and controlling cell migration. Then ELMO-1 binding Dock180 activated the Rac-GEF pathway, which contributed to the rearrangement of microtubules (Grimsley et al. 2004; Gumienny et al.

Table 1 ELMO-1 genetic variant rs741301 associated with T2DM and diabetic nephropathy

Author	Subject	Genotyping Methods	Risk allele (non-risk allele)	OR (95% CI
Shimazaki et al. (2006)	Japanese, 466 T2DM DN as case group and 266 T2DM non-DN as a control group.	DNA sequencing and TaqMan assay	G (A)	2.67(1.71–4.16)
Mooyaart et al. (2011)	Asian population	Meta-analysis of 2 studies in Asia population involved 546 T2DM with DN and 334 T2DM without DN		1.58 (1.28–1.94)
Wu et al. (2013)	Jiangzou Chinese, 123 T2DM with DN and 77 without DN.	MassARRAY	A (G)	1.89 (1.22–2.95)
Yadav et al. (2014)	North Indians, 215 T2DM, 202 T2DM with DN and 197 healthy control.	Taqman allele discrimination assay	G (A)	1.43 (1.1–1.88) for T2DM but not statistically significant as a risk for DN.
Mehrabzadeh et al. (2016)	Iranian, 100 T2DM without DN, 100 with DN and 100 healthy control.	Tetra ARMS PCR	G (A)	1.7 (1.17–2.63)
Bodhini et al. (2016)	South Indian population, 583 T2DM with DN and 601 T2DM without DN	MassARRAY system	A (G)	1.75 (1.20–2.55) for AG 1.48 (1.02–2.55) for GG
Hou et al. (2019)	Chinese Han, 660 T2DM with DN and 665 T2DM without DN	PCR-RFLP	G (A)	1.75 (1.19–2.28)
Bayoumy et al. (2019)	Egyptian, 200 T2DM with DN, 200 T2DM without DN and 100 healthy control	RT PCR allele discrimination technique	G (A)	1.82 (1.12–3.41) for DN in T2DM not statistically significant for risk of T2DM.
Yahya et al. (2019)	652 Chinese, Malays, and Indians reside in Negeri Sembilan Malaysia, suffering T2DM with or without DN.	MassARRAY system		Not statistically significant as a risk for DN in T2DM

(continued)

Table 1 (continued)

Author	Subject	Genotyping Methods	Risk allele (non-risk allele)	OR (95% CI
Omar et al. (2021)	Egyptian, 100 T2DM with DKD, 102 T2DM without DN and 102 healthy control	RT PCR allele discrimination technique	G (A)	2.366 (1.450–3.859) for T2DM but not statistically significant as a risk for DN.

ARM-PCR: Amplification-refractory mutation system- polymerase chain reaction; CI: Confident interval; DN: Diabetic nephropathy; OR: Odds ratio; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; RT-PCR: Real-Time PCR; T2DM: Type 2 Diabetes mellitus

2001). Independent of that pathway, Shimazaki et al. 2006 reported the role of this protein in DN pathophysiology, which contributed to excess deposition of extracellular matrix and lack of cell adhesive to ECM (Shimazaki et al. 2006).

Rat model for chronic glomerulonephritis showed increased expression of ELMO-1 protein in renal cortex and glomeruli than the control without chronic glomerulonephritis. Modified COS cell culture, which has ELMO-1 overexpression, showed increased fibronectin and integrin like kinase (ILK). These two proteins are components of the deposition of the ECM and adhesive cell propertied to ECM. Furthermore, the COS cells were transfected with siRNA targeting ELMO-1 and ILK having fibronectin suppression (Shimazaki et al. 2006).

Another study used genetically modified Akita mice (type 1 diabetes model with hyperglycemia) with various grades of systemic ELMO-1 (30–200%) in all tissue. They reported renal function and renal histopathology appearance, including albuminuria, glomerulosclerosis and glomerular base membrane (GBM) thickening worse parallel with increased expression of ELMO-1. This study reported it was influenced by increasing expression of TGF-ß, endothelin-1 and marker of stress oxidative NADPH oxidase-4 and reduced erythrocytes level of glutathione (Hathaway et al. 2016). This event might be through the ELMO-1 activated Rac pathway in vascular-related diseases, which induced increased stress oxidative by activation NAD(P)H oxidase (Kakoki et al. 2019; Yoshida et al. 2014). The study mention before also reported the ELMO-1 level to 30% sufficient to prevent glomerular injury caused by hyperglycemia. The ELMO-1 has potency as a DN therapeutic marker and even therapy target (Hathaway et al. 2016).

In addition, ELMO-1 protein inhibits cell adhesion into the extracellular matrix, altering effacement of podocytes foot process, increased apoptosis podocytes associated with altering of glomerular filtration barrier (Hathaway et al. 2016; Sharma et al. 2016). Those pathology associated with decline renal function in DN. Zebrafish model for hyperglycemia showed the detrimental effect of their renal analogue organ due to loss of podocytes cell caused by increased apoptosis, effacement podocytes foot process associated with loss of glomerular barrier. This event rescue with genetic modified targeting ELMO-1 overexpression and inhibitor of Pancaspase (Sharma et al. 2016). All the studies summary present in Table 3.

Table 2 ELMO-1 genetic variant located in various intron associated with diabetic nephropathy

Author	Subject	Genotyping Methods	SNP	Risk allele (non-risk allele)	OR (95% CI
Leak et al. (2009)	African American, 1135 T2DM-ESRD as case group and 1160 non-diabetic control consist of 2 set groups.	DNA sequencing	Intron 13 rs1345365	G (A)	The odds ratio for minor allele as protective allele between 0.77–0.84
			Intron 13 rs1981740	C (A)	
			Intron 13 rs10951509	G (A)	
			Intron 13 rs2058730	T (C)	
Hanson et al. (2010)	American Indian, 141 T2DM with nephropathy and 416 without nephropathy in a family study of 257 sibships. The ESRD subject was 107 cases, and 108 subject controls not suffering nephropathy	AD-PCR in conjunction with the 7000 Sequence Detection System or the iPLEX assay.	Intron 13 rs1345365	A (G)	2.42 (1.35–4.32)
			Intron 13 rs10951509	A (G)	2.42 (1.31–4.48)
Kim et al. (2010)	Mexican American, 455 T2Dm with DN and 437 T2DM without DN	Not mention clearly	46 SNP in ELMO-1 gene		Not statistically significant as the risk of DN in T2DM
Turki et al. (2018)	Tunisian Arabs, 600 patients with normoglycemic as control and 900 T2DM patients	PCR-RFLP	Intron 20 rs7782979	A (C)	The higher percentage of AA and CA genotype in T2DM than CC genotype in males subject
Hou et al. (2019)	Chinese Han, 660 T2DM with DN and 665 T2DM without DN	PCR-RFLP	3′ flanking-region rs10255208	G (A)	1.41 (1.06–1.92)
			Intron 13 rs1345365	G (A)	1.24 (0.77–1.95)
			Intron 20 rs7782979	A (C)	1.23 (0.82–1.76)

AD-PCR: Allelic discrimination PCR; CI: Confident interval; DN: Diabetic nephropathy; OR: Odds ratio; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; T2DM: Type 2 Diabetes mellitus

Table 3 The ELMO-1 plays a role in DN progression; studies in the animal model and cell culture

Author	Subject	Methods	Result
Shimazaki et al. (2006)	Rat model for chronic glomerulonephritis (uni-nephrectomy with the injection of anti-Thy1.1 antibody	In situ hybridization	The expression of ELMO-1 significantly increased in the renal cortex and glomeruli in the rat model for chronic glomerulonephritis than control mouse model.
	Modified COS cells that have overexpression of ELMO1 and siRNA targeting ELMO-1	RT-PCR, ELISA, western blot and cell-adhesion assay	In COS cells which overexpressed ELMO-1 increased fibronectin, integrin-linked kinase (ILK) expression and inhibited cell adhesion to the extracellular matrix. The COS cells, which transfected siRNA targeting ELMO-1and ILK, caused fibronectin suppression.
Sharma et al. (2016)	Genetically modified zebrafish model with PDX1 knockdown (hyperglycemia model) and CRISPR/Cas9 (knockout) of ELMO-1.	RT PCR, TUNEL assay	The animal model caused the detrimental effect of pronephric structure and function, podocytes foot processes, glomerular filtration barrier. Those conditions significantly rescue via ELMO-1 overexpression and Pancaspase inhibitor.
Hathaway et al. (2016)	Genetic modified ($Ins^{2Akita)}$ mice model for DM which have various grades of ELMO-1 in all tissue (30–200%)	RT PCR, not to mention methods for plasma protein analysis and glutathione.	Albuminuria, glomerulosclerosis and GBM thickening severity increased in parallel with ELMO-1 expression. Parallel with that event, this study reported an increase of TGF-ß, endothelin-1, NADPH oxidase-4 gene expression. In addition, the increased plasma level of cystatin C, lipid peroxide, TGF-ß and reduced erythrocytes level of glutathione also founded.

ELISA: Enzyme-linked immunosorbent assay; GBM: Glomerular base membrane; NADPH: Nicotinamide adenine dinucleotide; RT-PCR: Real-Time PCR; TGF Transforming growth factor; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

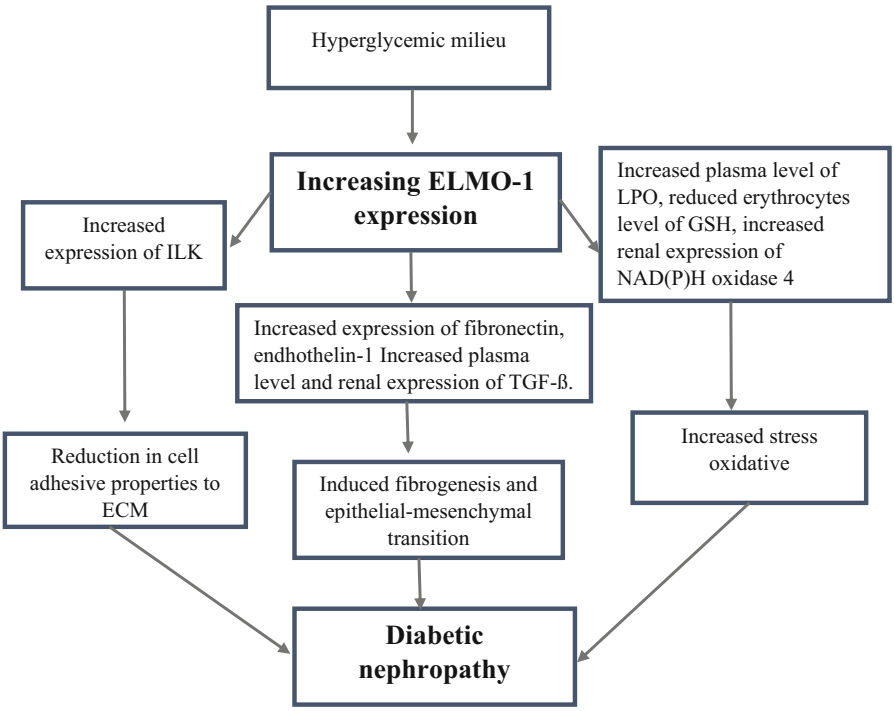

Fig. 1 Increased ELMO-1 expression in hyperglycemia milieu induced diabetic nephropathy. Hyperglycemia milieu in diabetes mellitus induced increasing ELMO-1 expression. The increasing ELMO-1 induced increasing expression marker of fibrogenesis and epithelial-mesenchymal transition, stress oxidative and reduction cell adhesive to ECM. These cellular processes lead to diabetic nephropathy

In summary, hyperglycemia milieu, which occurred in T2DM, induced increased expression of ELMO-1 protein. The increased ELMO-1 induced marker of fibrogenesis, epithelial-mesenchymal transition, stress oxidative, and reduction cell adhesive propertied to ECM. These processes explain the biological plausibility of ELMO-1 protein plays a role in diabetic nephropathy (Fig. 1).

ELMO-1 in Circulation and Renal Tissue in Humans as a Potential Biomarker of DN in T2DM

To their best of authors literature searching, studies ELMO-1 protein in human-related to DN still limited, summaries in Table 4. The study by Sharma et al. 2016 reported that the expression of ELMO-1 in renal of diabetic, non-diabetic and polycystic kidneys did not significantly differ. Podocytes, tubules, and glomerular

Table 4 The ELMO-1 studies in human-associated with DN

Author	Subject	Methods	Result
Sharma et al. (2016)	Kidney section from non-diabetic (n = 5), diabetic type 2 (n = 5) and polycystic kidney diseases (n = 5)	IHC and fluoro-IHC	ELMO-1 is expressed in podocytes, tubules and is almost absent in glomerular endothelial cells. ELMO-1 expression remained unchanged within the kidney of T2DM and polycystic diseases.
Elfiani et al. (2020)	Plasma of T2DM with various albuminuria grade. Non albuminuria (n = 20), microalbuminuria (n = 20), macroalbuminuria (n = 20)	Sandwich ELISA for human ELMO-1	The macroalbuminuria patients have higher plasma ELMO-1 than microalbuminuria. Plasma ELMO-1 had a positive correlation with ACR. Plasma ELMO-1 had a negative correlation with GFR.

ELISA: Enzyme-linked immunosorbent assay; IHC: Immuno-histopathology Chemistry; T2DM: Type 2 Diabetes Mellitus

endothelial cells express the ELMO-1. The limitation of this study was limited number of kidney sections were involved, and not known if the renal diabetic sample was suffering DN (n = 15) (Sharma et al. 2016).

Another study performed by Elfiani et al. 2020 reported plasma levels of ELMO-1 to have a positive correlation with ACR and a negative correlation with GFR in T2DM. In addition, significantly higher levels of plasma ELMO-1 in macroalbuminuria than microalbuminuria or normoalbuminuria (Elfiani et al. 2020). The plasma level of ELMO-1 was not correlated with plasma glucose level, different from with previous in-vitro study which reported high glucose enhanced ELMO-1 expression in cell culture (Shimazaki et al. 2006).

Application to Prognosis, Other Diseases or Condition

This chapter review studies of ELMO-1 in genomic, transcriptomic and proteomic level associated with DN in T2DM in the human, animal model and cell line. The genetic variant of rs741301 may be applied as risk screening in T2DM patients to predict DN. It can apply in Asia and Europe but not in American (Bodhini et al. 2016; Hou et al. 2019; Kim et al. n.d.; Mehrabzadeh et al. 2016; Mooyaart et al. 2011; Shimazaki et al. 2005; Wu et al. 2013; Yadav et al. 2014). The ELMO-1 genetic variant that tends to be associated with DN in the American population is the genetic variant located at intron 13 (Hanson et al. 2010; Leak et al. 2009). Furthermore, increased systemic ELMO-1 level in the animal model and cell line parallel with increased severity of histopathology and decline renal function marker associated with DN. Those studies reported events associated with increased excess ECM deposition and oxidative stress marker, two major pathways in DN pathophysiology (Hathaway et al. 2016; Sharma et al. 2016; Shimazaki et al. 2006). To the best of our

Table 5 Insight of ELMO-1 discoveries biomarker for DN

Biomarker criteria	Research was performed	Further research needed
Non-invasive, easy to measure, inexpensive, readily available sources	As shown by Elfiani et al., the sample for measure ELMO-1 was plasma with ELISA in the previous study. As a summary in Table 3, systemic ELMO-1 plasma level reflected renal changes in DN.	Further research with the urinary sample was needed.
High sensitivity and specificity to detect early renal lesion of DN	The data is not yet available.	Further research with more significant sample number and multicenter, compared with other and gold standard of plasma ELMO-1 as a biomarker of DN
Able to treatment monitoring, diseases risk stratification and possess prognostic value	As shown by Hathaway et al., the animal study reported that grading of ELMO-1 expression parallel with worsening of DN marker.	Further study in humans which specific cut of point of ELMO-1 for monitoring and predict prognostic of DN
Biologically plausible	Summary in Fig. 1	Another biology plausible of ELMO-1. It might be the role of ELMO-1 in immunology response related to apoptotic and immune cell migration caused by DN pathology changes.

literature searching, one study in the human reported a correlation of ELMO-1 with two typical decline renal function markers (ACR and estimated GFR) (Elfiani et al. 2020). The previous studies above strengthen the evidence that ELMO-1 may apply as a biomarker of DN progression in T2DM. The circulating ELMO-1 might be used as a DN progression biomarker, although further studies are still needed to elucidate if the ELMO-1 meet the ideal biomarker for monitoring DN progression.

Answering the question is ELMO-1 a DN progression biomarker in T2DM, the author tried their best to conclude based on previous studies written before (Table 5). Previous studies measure the plasma level and renal expression of ELMO-1, which both showed can represent the DN progression (Elfiani et al. 2020; Hathaway et al. 2016). In addition, the previous studies have proven the biology plausibility of ELMO-1 in DN progression, which is in summary in Fig. 1 (Hathaway et al. 2016; Sharma et al. 2016; Shimazaki et al. 2006). The methods used are familiar but need specific laboratories resources. Since these methods are not used widely, the price is relatively high (Tabels 1, 2 and 3). The study mentioned above is a preclinical study that cannot yet compare the sensitivity and specificity of ELMO-1 with other biomarkers. The ELMO-1 might be able to monitor the severity of the diseases, treatment value, and prognostic value showed as reported by genetically

modified animal study with various grades of ELMO-1 expression showed various grades of histopathology and renal function marker (Hathaway et al. 2016). This gene was reported as a genetic marker of DN (Table 1). Further research in humans which investigated the sensitivity and specificity of this biomarker for DN progression was needed.

Mini-Dictionary

Albuminuria is the amount of albumin that appears in the urine, the albumin normally not passed the glomerular barrier.

Estimated GFR is the estimated rate of glomerular filtrate blood, measured with a specific equation mainly based on serum creatinine.

Genetic variant is changes of DNA sequences that may affect protein which encodes.

Diabetic nephropathy is a disease associated with renal structural and functional changes associated with diabetes.

Extracellular matrix is a non-cellular component present within the tissue which involved in cellular homeostasis.

Key Facts

Key Facts of Albuminuria

- Albuminuria is widely used as a clinical marker for diabetic nephropathy progression.
- Progression of normo-albuminuria to micro-albuminuria and macro-albuminuria reflected progression to worse of DN progression.
- Unfortunately, the accuracy of this biomarker as DN progression become doubtful.
- A previous study reported that 21–62% of T2DM patients with microalbuminuria regress to albuminuria.
- In addition, more than 30% patient with decline GFR has normo-albuminuria.
- Normo-albuminuria patients have various grading of typical histopathology appearance reflected of DN

Key Facts of ELMO-1 Protein

- ELMO-1, known as protein, plays a role in cell viability and migration.
- A study with cell lines reported increased ELMO-1 expression in hyperglycemia conditions.
- Further study showed increased plasma level and tissue level of ELMO-1 associated with DN progression.

- This protein is expressed in glomerular, podocytes cells, and tubular parts of renal tissue.
- Excess deposition of extracellular matrix and stress oxidative were found parallel with increased ELMO-1 in DN progression.

Key Facts of ELMO-1 Genetic Variant rs741301

- This genetic variant is located in the non-coding region of the ELMO-1 gene, in which adenosine (A) substitute guanosine (G).
- This genetic variant may affect protein transcription and protein stability although located in a non-coding region (intron).
- Genotype and phenotype association studies in various populations reported this genetic variant as the risk for DN susceptibility in T2DM patients.
- Patients who have risk allele are more susceptible 1.43–2.67 times for suffering DN than non-risk allele.
- The risk allele was different beyond the population. The G allele more pronounces as a risk allele than the A allele.

Summary Points

1. ELMO-1 genetic variant rs741301 associated with susceptibility for suffering DN in Asian and European populations.
2. ELMO-1 genetic variant located in intron 13 associated with susceptibility for suffering DN in the American population
3. Increased system ELMO-1 associated with severity of DN in T2DM through excess deposition of ECM, decreased cell adhesive properties to ECM, increased stress oxidative, influence podocytes apoptosis, and foot process effacement in the animal model and cell line.
4. Plasma ELMO-1 at the protein level have potency as a biomarker that can predict the severity of DN in T2DM patients. It is associated with a decline in renal function markers.
5. Further study in a human was needed to strengthen the evidence of using ELMO-1 as a biomarker to treat DN.

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Brain-Derived Neurotrophic Factor and Vascular Endothelial Growth Factor A: Biomarkers Potential in Diabetes

23

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Contents

Introduction 487
Brain-Derived Neurotrophic Factor: Mechanisms of Action and Functions 487
Vascular Endothelial Growth Factor A: Mechanisms of Action and Functions 489
Diagnostic and Therapeutic Potential of Brain-Derived Neurotrophic Factor in Metabolic Disturbances of Type 2 Diabetes Mellitus 490
Study of Serum Concentrations of BDNF in Human Populations 493
Inflammation and Brain-Derived Neurotrophic Factor 494
Diagnostic and Therapeutic Potential of Vascular Endothelial Growth Factor and Type 2 Diabetes Mellitus 495
Type 1 Diabetes Mellitus and Growth Factors 496
Serum BDNF and VEGFA as Biomarkers of Diabetes Complications 497
Study of Serum Concentrations of BDNF and VEGFA in Human Populations in Diabetic Polyneuropathy 499
Differences in Serum Levels of Neurotrophins in Diabetic Polyneuropathy in Type 1 and Type 2 Diabetes Mellitus 502
Modulation of Neuropathic Pain by BDNF in Diabetic Neuropathy 503
Central Nervous System Disturbances in DM: The Role of Growth Factors 503
Applications to Prognosis, Other Diseases, or Conditions 504
Key Facts of Brain-Derived Neurotrophic Factor and Vascular Endothelial Growth Factor A: Biomarkers' Potential in Diabetes 505
Mini-Dictionary of Terms 506
Summary Points 506
References 507

Abstract

Previously, brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) have been studied only in diabetic neuropathy or vascular

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complications in diabetes. Recent numerous studies have investigated their role in metabolic control, inflammatory response, and involvement in reducing insulin resistance, leptin levels, and even glycemic regulation as direct points of the multifaceted pathogenesis of diabetes mellitus. Since these growth factors are widely produced by various types of tissues and organs, BDNF and VEGF are highly sensitive to damage of tissues involved in the pathogenesis of diabetes. Thus, serum levels of BDNF and VEGF can be considered as valid biomarkers of diabetes mellitus. However, there are still many open questions, for instance, the specific levels of the factors in human serum at different stages of diabetes and its complications, the certain role in formation of obesity, and neuropathic pain. Factors capable of altering serum neurotrophin concentrations remain unexplored. In addition, current medicine requires researches devoted to abilities to modulate intracellular reactions triggered by the interaction of neurotrophins and their receptors, as a treatment for neuropathic pain, restoration of nerve fibers and other tissues, as well as regulation of glycemic control, bypassing the side negative proliferative reactions of hyperactivation of growth factors.

Keywords

Brain-derived neurotrophic factor · Vascular endothelial growth factor · Diabetes mellitus · Diabetic polyneuropathy · Tropomyosin-related kinase B receptor · Vascular endothelial growth factor receptor 2

Abbreviations

AKT	RAC-alpha serine/threonine-protein kinase
BAD	BCL2-associated agonist of cell death
BDNF	Brain-derived neurotrophic factor
Bcl-2	B-cell lymphoma 2
B-Raf	Kinase signaling pathway MAPK/ERK
CaMKII	Calmodulin-dependent protein kinase II
DNA	Deoxyribonucleic acid
c-Fos	Pro-oncogenic protein Fos
c-Myc	Pro-oncogenic protein Myc
CREB	Cyclic AMP-sensitive bound protein
CRP	C-reactive protein
ENMG	Electroneuromyography
ERK	Extracellular signal-regulated kinase
GSK-3b	Glycogen synthase kinase 3b
GABA	Gammaaminophenylbutyric acid
IP3	Inositol triphosphate
MAPK	Mitogen-activated protein kinase
m-TOR	Mammalian target of rapamycin
NGF	Nerve growth factor
PI3K	Phosphoinositide 3-kinases
PLCγ	Phosphoinositide phospholipase C

TrkB	Tropomyosin-related kinase B receptor
VEGFA	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor 2

Introduction

Growth factors are integral participants in pathogenesis of diabetic polyneuropathy as basic mechanisms of nervous system adaptation to pathological conditions. Brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor A (VEGFA) have been studied quite well in a variety of diseases, including both central and peripheral nervous systems and cardiovascular disorders. The pathogenesis of diabetes implies damage to the nervous system and the development of microangiopathic complications from the earliest stages. Given these data, these neurotrophins can be valuable in predicting diabetes and its complications.

Brain-Derived Neurotrophic Factor: Mechanisms of Action and Functions

Brain-derived neurotrophic factor is a protein belonging to the neurotrophin family. The mechanisms of its action have been studied in sufficient detail and used in the study of the pathogenesis of many diseases of a nervous system. The main functions of a brain-derived neurotrophin in peripheral nervous system (PNS) are the prevention of apoptosis and the provision of regenerative processes, in particular the induction of dendritic branching and axonal sprouting in the direction of target cells (Benarroch 2015). In the central nervous system (CNS), BDNF regulates synaptic transmission, including both rapid signal transmission and the phenomenon of long-term potentiation cognitive functions (Lamb et al. 2015). In addition BDNF ensures the maintenance of viability, differentiation, and proliferation of neurons of various neurotransmitter modalities (dopaminergic, cholinergic, GABA and glutamatergic, serotonergic) (Numakawa et al. 2018).

Neuroplasticity effects of BDNF are mediated by its interaction with the highly specific tropomyosin-related kinase B (TrkB) receptor. This receptor contains an intracellular protein kinase domain, ligand-binding leucine-rich extracellular repeats, and transmembrane immunoglobulin-like domains (Sasi et al. 2017). The receptor is produced by structures of both CNS and PNS and is found in the brain cortex, hippocampus, brain stem, thalamus, retina, and spinal cord, along with spinal and cranial ganglia and Meissner's corpuscles (Gupta et al. 2013). In addition to the nervous system, the receptor has been isolated in skeletal muscle, kidney, and pancreas.

Integration of BDNF and its TrkB receptor mediates intracellular signaling cascades of interrelated responses, as shown in Fig. 1. One of the main signaling pathways triggered by the neurotrophin receptor system is the ERK (Ras-ERK, MAPK / ERK) pathway: the key regulatory subunit Shc activates the expression

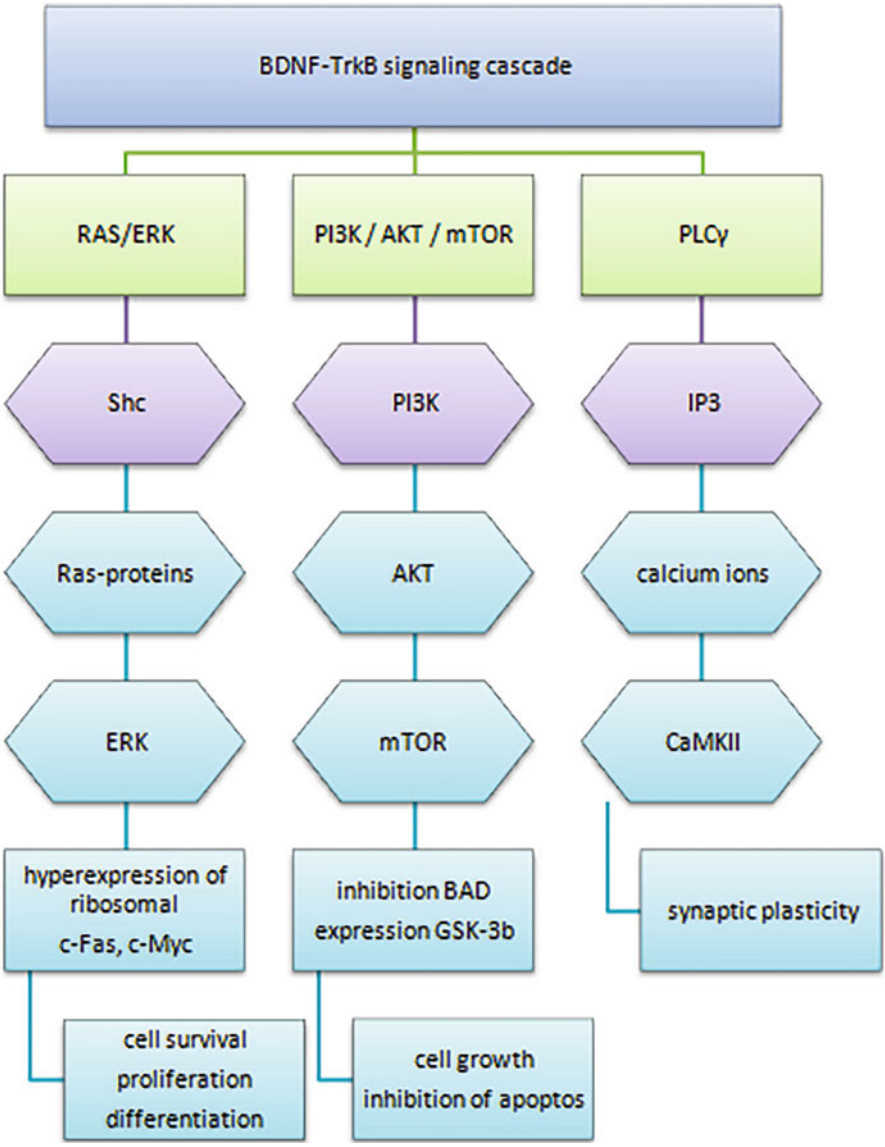

Fig. 1 Cascade of intracellular signalling reactions mediated by an interaction between BDNF and TrkB

of the Ras membrane protein, which includes the sequential activation of various MAPK/ERK kinases, which ultimately activates the ERK1/2 enzyme, which diffuses into the cytoplasm, where it interacts with signaling kinases of ribosomal proteins, subsequently penetrating into the nucleus, where it induces the transcription of the c-Fos and c-Myc genes, the products of which are responsible for cell

proliferation, survival, and differentiation (Guo et al. 2018). The second signaling pathway activated by Shc-protein is the PI3K/AKT/mTOR signaling pathway. Under the action of the enzyme phosphatidylinositol-3-kinase (PI3K), AKT kinase and m-TOR (mammalian target of rapamycin) protein responsible for the cell cycle at different phases and the organization of the actin cytoskeleton are activated. The activity of GSK-3b (glycogen synthase kinase 3b), a protein necessary for survival, is increased; in addition, the content of BAD, a functional agonist of Bcl-2 (B-cell lymphoma 2), a promoter of apoptosis and cell death, decreases (Yu and Cui 2016). The PI3K/AKT/mTOR signaling pathway is necessary for cell growth and metabolism and inhibits apoptosis. The third major mechanism of the BDNF/TrkB cascade is the activation of phosphatidylinositol-specific phospholipase C (PLCγ). Through the formation of IP3 from PLCγ, calcium ions are released from the intracellular pool, which triggers important calcium-dependent reactions, in particular the activation of calmodulin-dependent protein kinase CaMKII, the main regulator of synaptic plasticity (Guo et al. 2018). This mechanism is necessary for long-term potentiation, which is the basis of memory.

However, the expression and, accordingly, the zones of influence of brain-derived neurotrophic factor are not confined by central and peripheral systems only. The main non-neuronal structures expressing BDNF are skeletal muscles, activated immune cells, endothelial and liver cells, and adipocytes. BDNF biosynthesis occurs in human megakaryocytes; subsequently, the factor is accumulated and expressed by platelets and is found in serum (Sasi et al. 2017). Taking into account the wide expression of BDNF, this factor has many zones of influence and additional functions and, due to this, is a potential biomarker of pathological processes involving the tissues synthesizing it. In particular, its role in carbohydrate metabolism and energy homeostasis in diabetes mellitus is being actively studied.

Vascular Endothelial Growth Factor A: Mechanisms of Action and Functions

Vascular endothelial growth factor A (VEGFA) was described as the first member in the family of vascular endothelial growth factor. Later, VEGF-B, involved in embryonic angiogenesis; VEGF-C and VEGF-D, responsible for lymphangiogenesis; and placenta growth factor (PIGF) were identified. This factor is expressed mainly by endothelial cells, as the name suggests, but monocytes, macrophages, platelets, neurons, nephrocytes, myofibroblasts, and epithelial cells can also produce it. Expression of vasculoendothelial growth factor occurs in response to hypoxia, as well as under the influence of pro-oncogenes, pro-inflammatory cytokines (TGF-alpha, TNF-alpha, fibroblast growth factor (bFGF), interleukin-1), and matrix metalloproteinases (MMP) (Vempati et al. 2014).

Three main receptors with tyrosine kinase activity have been identified for vasculoendothelial growth factor – VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-2 appears to mediate almost all of the observed responses of endothelial cells to VEGF, while the type 1 receptor has a ligand-binding role for signaling

through VEGFR-2 (Peach et al. 2018). These receptors can exist in both transmembrane and free form. In addition, free forms are considered as endogenous inhibitors of the action of vascular endothelial factor, since the ligand enters the "trap" in the serum, does not reach the membrane receptors of the endothelial cells, and cannot carry out a cascade of intracellular signaling responses for survival (Ebos et al. 2004).

Similar to TrkB activation, after ligand binding, the intracellular portion of the VEGFR2 receptor dimerizes and undergoes a phosphorylation reaction, as illustrated in Fig. 2. Unlike other types of tyrosine kinase receptors, which mainly use the RAS activation pathway, VEGFR2 acts to a greater extent through the activation of phosphatidylinositol-3-kinase (PI3K) (Shibuya 2011). Activation of this signaling pathway promotes the survival of endothelial cells by inhibiting apoptosis, enhancing the antioxidant defense of the cell, and potentiating DNA synthesis, while the cascade of signaling reactions mediated by the activation of Shc2 and MAP kinases induces the proliferation of endothelial cells and arteriogenesis (Guo et al. 2017). In addition, VEGF interacts with endothelial NO synthase (eNOS) and enhances NO production, promoting vasodilation.

The activity of vascular endothelial growth factor has been studied most widely in the functioning of the cardiovascular system (Cahill and Redmond 2016). Originally discovered as the main triggering mechanism of angiogenesis, VEGF has shown itself to be a factor in the protection and restoration of cardiomyocytes in myocardial infarction, ensuring collateral circulation in ischemic strokes, and vascular remodeling in arterial hypertension. An increase in the expression of free receptor and VEGF in serum has been demonstrated during the progression of chronic heart failure. Vascular growth factor potentiates migration and division of endothelial cells, provides chemotaxis of macrophages, and enhances NO expression and, accordingly, vasodilating properties. In addition, VEGF promotes hyperpermeability of endothelial cells, which leads to profound changes in the extracellular matrix and creates even more favorable conditions for angiogenesis.

The role of vasculoendothelial growth factor in dyslipidemia and in the progression of atherosclerosis was studied. Oxidized low-density lipoproteins are absorbed by macrophages and initiate the formation of foam cells. Given the incidence of micro- and macroangiopathic complications in type 2 diabetes, this factor may be a potential marker for the development of diabetes and its vascular manifestations.

Diagnostic and Therapeutic Potential of Brain-Derived Neurotrophic Factor in Metabolic Disturbances of Type 2 Diabetes Mellitus

The history of the study of growth factors in diabetes mellitus begins from experimental neurological studies demonstrating good regenerative capabilities of nerve growth factor and its family of neurotrophins in various damages of peripheral nervous system (Foster et al. 1994). This knowledge could not fail to suggest the need to study growth factors in diabetic polyneuropathy from the standpoint of

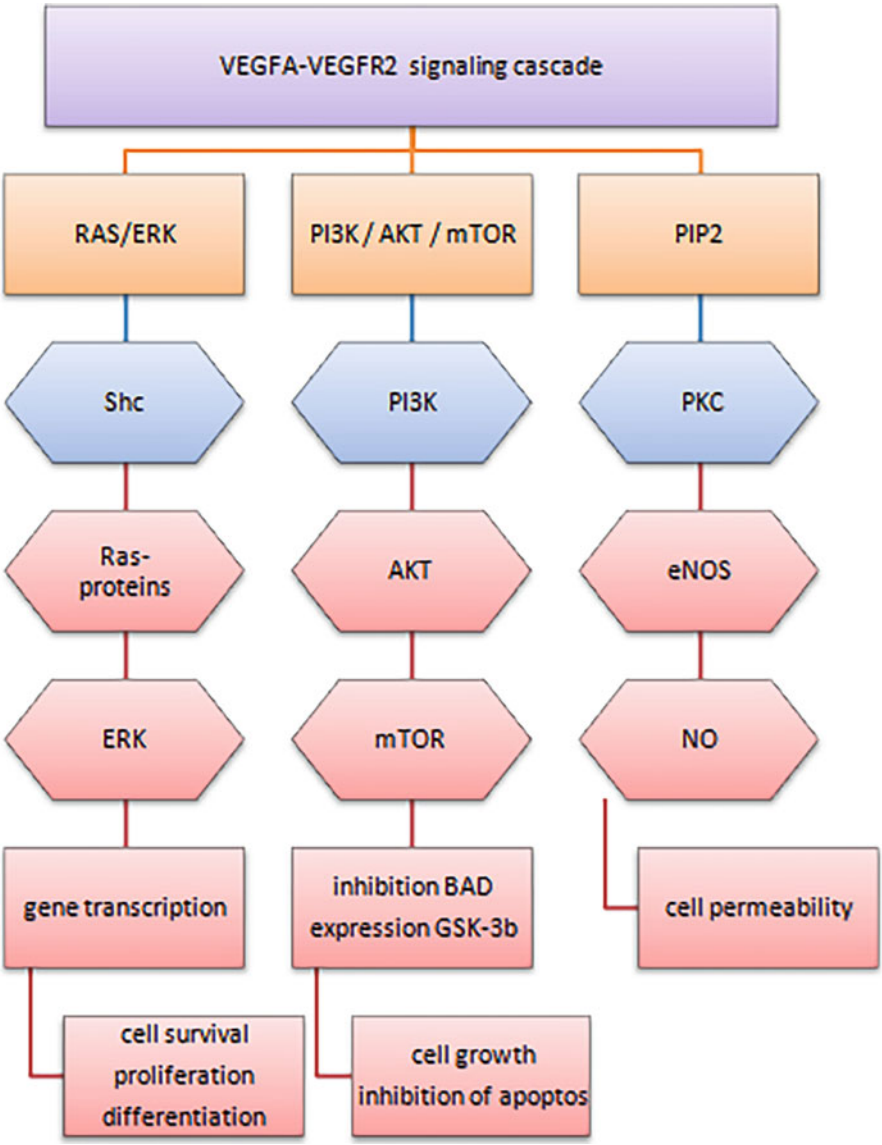

Fig. 2 Cascade of intracellular signalling reactions mediated by an interaction between VEGF and VEGFR2

damage and restoration of nerve fibers. Later, the hypothesis was confirmed in several studies (Apfel 1999; Kennedy et al. 1998).

In subsequent studies, attempts to find the relationship between metabolic disorders in diabetes and the expression of brain-derived neurotrophic factor were made. T. Nakagawa and coauthors put forward a new hypothesis that BDNF has endocrine

capabilities, in particular can decrease blood glucose and hyperphagia in experimental rat models with obesity. The authors describe the hypoglycemic effect of BDNF was more pronounced in young rodent with hyperinsulinemia and that BDNF enhanced the hypoglycemic effect of insulin in mice with streptozocin-induced diabetes. Afterwards, it was suggested that the implementation of the hypoglycemic activity of BDNF requires the participation of endogenous or exogenous insulin (Nakagawa et al. 2002).

The involvement of BDNF in metabolic control is not limited to the relationship with insulin and glucose: there is evidence of mutual regulation with the production of leptin, an active hormone-regulating eating behavior. In rat models with leptin receptor deficiency, administration of BDNF reduced hyperphagia and hyperglycemia (Tonra et al. 1999; Ono et al. 2000). At the same time, another group of Japanese researchers (Maekawa et al. 2013) demonstrated that low BDNF production in the ventromedial hypothalamus is associated with blood glucose levels, increased leptin secretion, and visceral fat mass in a rat model with type 2 diabetes mellitus (T2DM). In their study, BDNF administration significantly reduced plasma leptin levels for a long time in hyperleptinemic T2DM rats. Komori T. and coauthors also reported that BDNF synthesis is inhibited by leptin (Komori et al. 2006). Thus, BDNF dysfunction is integral part in counteracting hyperleptinemia, leptin resistance, and improving central regulation of energy metabolism in obesity.

Experimental studies have demonstrated a beneficial effect on reducing insulin resistance in laboratory animals. Laboratory mice with streptozocin-induced diabetes mellitus received intracerebral injections of BDNF into the nucleus of the hypothalamus, which reduced hyperglycemia. However, in an experiment on obese animals without diabetes, a similar effect (improvement of glycemia) was not observed (Ono et al. 1997). This may indicate an indirect effect of BDNF on the normalization of carbohydrate metabolism by reducing insulin resistance in peripheral tissues.

In a study of Kuroda A on obese laboratory rats, which also received BDNF injections, a decrease in fasting and postprandial glycemia levels was demonstrated, and the mechanism was also described in detail – exogenous BDNF is able to increase the expression of hepatic glucokinase, which reduces gluconeogenesis and decreases insulin resistance (Kuroda et al. 2003).

Another study showed the BDNF protective effect for pancreatic beta cells: Yamanaka M et al. studied mice with obesity and diabetes, one group received injections of BDNF, the second – a placebo. According to the results of the study, the first group was characterized by a lower concentration of glucose, a lower concentration of pancreatic glucagon, and an increased level of pancreatic insulin and a histologically larger area of insulin-producing cells of the pancreas islet, compared with the placebo group (Yamanaka et al. 2008). In addition, the potential regenerative effect of brain-derived neurotrophin was discussed in the article by Hristova MG (2017), in particular due to the possibility of activation of the neurogenin gene by the BDNF, which subsequently leads to the regeneration of beta cells and increased insulin secretion.

Study of Serum Concentrations of BDNF in Human Populations

At the same time, experimental models of diabetes in animals cannot be considered unambiguously as representative models of diabetes mellitus and its complications for humans. The uncontrolled course of experimental diabetes in laboratory mice leads to their dehydration and severe catabolic state, as a result of which growth factors act more as local factors for canceling the catabolic stage in the affected limb than by changing conditions specifically associated with the multifactorial pathogenesis of diabetes mellitus in humans. Currently, clinical studies devoted the role of BDNF; VEGF in type 2 diabetes mellitus in human populations show conflicting data.

Thus, several studies have revealed a low concentration of BDNF in the serum of patients with type 2 diabetes compared with healthy controls (Krabbe et al. 2007; Li et al. 2016; Zhen et al. 2013; He and Wang 2014). These results were also observed by Fujinami A. and coauthors (Fujinami et al. 2008), who, in their study, moreover, demonstrated sex differences in concentrations: the group of women with type 2 diabetes was characterized by a higher level of BDNF compared to men with type 2 diabetes; however, in the healthy group, such differences were also found. The authors explain the obtained results by differences in estrogens, which can alter glucose metabolism. Indeed, several independent investigators have described the potential modulating effect of estradiol on the neurotrophin expression (Sohrabji et al. 1995). So, in an experimental study which studied mice subjected to oophorectomy, comparing treatment with estradiol and placebo, the group of mice treated with estradiol was characterized by greater expression of brain-derived neurotrophin than the control group (Yi et al. 2016). In addition, sex differences in the levels of BDNF can be interpreted by a key role of sex hormones in glucose metabolism and a modulating effect on the development of insulin resistance.

Several researchers obtained completely opposite results – they demonstrated higher levels of BDNF in serum in patients with type 2 diabetes mellitus than in healthy controls (Boyuk et al. 2014). At the same time, the control group in the study had a lower body weight, a smaller waist circumference, which could affect the results of the study.

Japanese researchers also obtained similar data: the level of BDNF was significantly higher in patients with type 2 diabetes than in the control group, correlated with BMI, subcutaneous fat area, and glycemic and triglyceride levels (Suwa et al. 2006). Opposite research data can be explained by the lack of a unified study design, heterogeneous samples, different severities of the disease, presence or absence of complications of diabetes mellitus, bad habits, lifestyle, weight, which can also change the concentration of neurotrophins, as well as the duration and severity of hyperglycemia, the presence of concomitant pathology and hormonal changes. At the moment, correlations between the duration of hyperglycemia and the decrease of neurotrophin concentration and the increase in insulin resistance are confirmed facts. Nevertheless, the question remains unclear, what is primary, increasing insulin resistance, which reduces the production of growth factors or a deficiency of neurotrophins, aggravates the severity of insulin resistance.

Inflammation and Brain-Derived Neurotrophic Factor

Systemic inflammation plays a central role in the pathogenesis of type 2 diabetes and its complications (Eyileten et al. 2017). The exact mechanisms are still not fully understood. The main donors of circulating inflammatory cytokines in diabetes are hepatocytes, macrophages, and adipocytes, an increase in the expression of interleukin-1β, interleukin-6, and acute phase markers such as c-reactive protein (CRP) (Pradhan et al. 2001). Several clinical studies have observed positive correlations between increased levels of brain-derived neurotrophic factor and increased expression of interleukin-6 in type 2 diabetes and in some other clinical diseases (Eyileten et al. 2016).

Krabbe K.S. and coauthors in their study determined the positive correlation between low levels of BDNF and CRP in patients with type 2 diabetes, regardless of obesity. However, in the control group such a correlation was not found (Krabbe et al. 2007). Thus, the connection between neurotrophin and inflammatory conditions, which play an important role in the progression of diabetes mellitus, is confirmed.

Currently, there is a scientific debate about the effect of hypercoagulation on the release of BDNF by platelets into plasma and, accordingly, plasma concentrations of BDNF. It has been proven that along with the state of hypercoagulation, with type 2 diabetes, platelet hyperreactivity occurs, which is most likely manifested in metabolic imbalance and systemic inflammation (Yazbek et al. 2003; Ferreiro et al. 2010).

In addition, in their study, Lorgis L and coauthors observed an increase in the level of BDNF and β-selectin, which is a biomarker of inflammation and platelet activation, in patients with myocardial infarction (Lorgis et al. 2010).

Also, research groups of Lim H. and Neubauer H. proposed to consider p-selectin levels as predictive markers of BDNF in patients with diabetes (Lim and Blann 2004; Neubauer et al. 2010).

Thus, altering serum neurotrophin's concentrations and considering them as diagnostic markers still have many pitfalls. In particular, a more in-depth study of the pathogenic and physiological aspects of type 2 diabetes is needed, changing the concentration of BDNF, as well as qualitative data generalization, such as systematic review and meta-analyses.

The only systematic review was performed by Davarpanah, M et al. (2021), who analyzed 16 of 167 studies and concluded that diabetic patients had lower serum BDNF levels than healthy controls. Correlations between the level of BDNF and glycemic parameters were not confirmed – these correlations were not considered in all studies included in the meta-analysis. This systematic review has a number of other limitations: the studies included in the review were of a heterogeneous design; researchers took into account a different number of potential factors that could affect the concentration of brain-derived neurotrophic factor; in addition, the sizes of study samples were small and differed in demographic parameters and the duration of diabetes mellitus. In some of the reviewed studies, BDNF was studied in blood plasma; in others it was detected in serum. The authors failed to conduct a gender

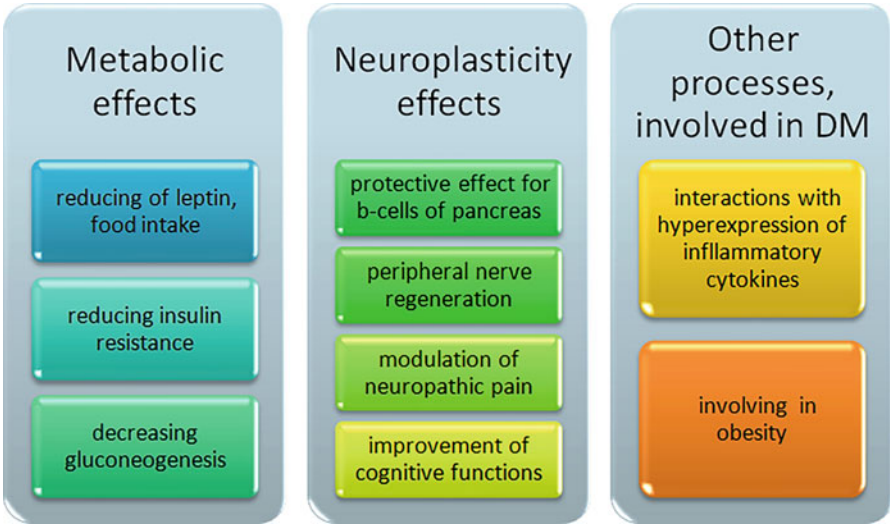

Fig. 3 Potential effects of BDNF in diabetes mellitus

stratified analysis. Thus, more standardized studies and further meta-analyses are needed to investigate the relationship between BDNF and DM in humans.

In their recent study, Furukawa et al. studied in more detail the role of altered levels of BDNF in the pathogenesis of type 2 diabetes mellitus. Researchers have studied the influence of one of the driving forces of the pathogenesis of hyperglycemia – the end products of glycation (AGE) – on the release of BDNF and came to the assumption that the expression of brain neurotrophin induced by AGE is a system of biological defense in the early stages of diabetes, and a chronic increase in AGE may result in depletion of BDNF reserves during the progression of diabetes (Furukawa et al. 2017).It is the first trying to explain the specific mechanisms of changes neurotrophins' concentrations in pathogenesis of DM.

The functions of BDNF in diabetes mellitus discussed in this and the next subchapters are schematically demonstrated in Fig. 3.

Diagnostic and Therapeutic Potential of Vascular Endothelial Growth Factor and Type 2 Diabetes Mellitus

Most studies devoted the role of VEGF in the development of diabetes mellitus, concerning primarily its macroangiopathic complications. According to basic research and systematic reviews, vascular endothelial growth factor appears as an important agent in the development and progression of endothelial dysfunction and vascular complications of diabetes (Mahdy et al. 2010).

It has been proven to increase VEGF in hyperlipidemia, which can provoke atherosclerotic processes in the vessels and aggravate the course of diabetes (Jiang

et al. 2019). Overexpression of VEGF, according to a number of studies, correlates with hypertension (Touyz et al. 2017).

In addition, it has been hypothesized the potential involvement of VEGF in the development of metabolic syndrome (Mazidi et al. 2017).

According to Zhang Q (Zhang et al. 2018), serum VEGF levels correlated with the level of glucose, glycated hemoglobin, and the percentage of T1-helpers, which also indicates the relationship of VEGF with inflammatory mechanisms and metabolic control.

The hypothesis that the relationship between VEGF and diabetes may be more complex and indirect than it seems is also held by a group of French researchers, who analyzed French and Danish populations regarding the variability of VEGF gene polymorphisms and the risks of diabetes: despite the dispersion of free VEGF circulating in the blood, no association was found between polymorphisms and the risk of type 2 diabetes and its complications (Bonnefond et al. 2013).

However, more and more current research confirms correlations between increased serum VEGFA levels, a glycemic control, an inflammation, and macroangiopathic complications (Hanefeld et al. 2016). In the Tunisian population, genetic polymorphisms of the VEGFA gene have been studied; it has been proven altered polymorphisms affect the expression of VEGFA and correlate with fasting glycemia, cholesterol, triglycerides, and glycated hemoglobin levels (Sellami et al. 2018).

The results of a recent study by Platania C. (Platania et al. 2019) are promising. The authors proved that overexpression of 8 dysregulated microRNAs affecting the expression of vasculoendothelial growth factor, brain-derived neurotrophin, and CREB1 occurs long before the onset of vasculopathy in the diabetic retina.

In a small study by Sugimoto K, skin sections were examined by confocal microscopy; the authors report the development of subepidermal microvascular proliferation and impaired VEGF production before the onset of overt clinical neuropathy, retinopathy, or nephropathy in patients with type 2 diabetes (Sugimoto et al. 2019).

Schematic representation of the role of the vascular endothelial factor in the development of diabetes and its complications is illustrated in Fig. 4.

Type 1 Diabetes Mellitus and Growth Factors

There is much less research evidence for type 1 diabetes and concentrations of growth factors. An experimental study demonstrated a decrease in the expression level of superoxide dismutase, a major component of antioxidant defense, and brain-derived neurotrophic factor in the brain of rats with type 1 diabetes (Xia et al. 2014). In patients with type 1 diabetes, the increased serum BDNF concentration was detected compared with healthy controls, and in both groups, the level of neurotrophin increased after exercise (Tonoli et al. 2015). However, in the population of Chinese children with type 1 diabetes, the decrease of serum BDNF was found, which correlated with poorer cognitive function and poor glycemic control (Chen et al. 2021).

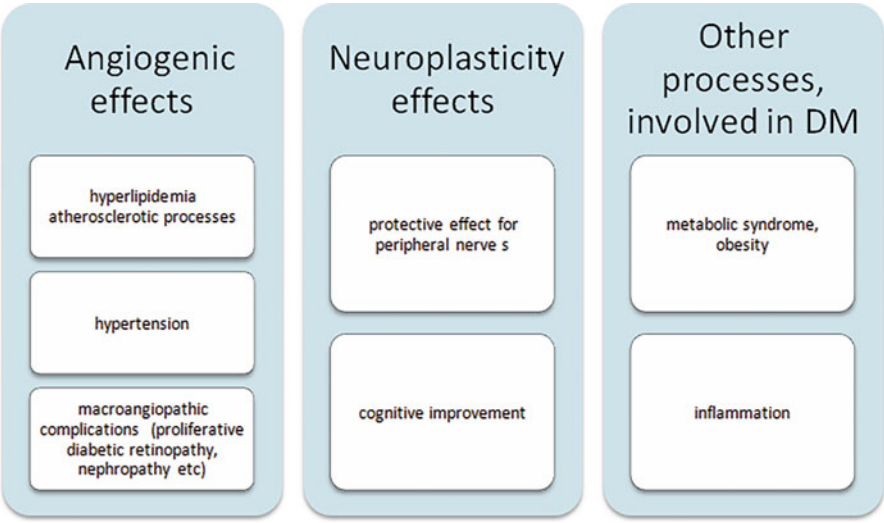

Fig. 4 Potential role of VEGFA in diabetes mellitus

Autoimmune and inflammatory processes leading to the destruction of the pancreas have a paramount importance in the pathogenesis of type 1 diabetes. A recent experimental study of Bathina S demonstrated a positive effect of the anti-inflammatory metabolite RVD1 on the course of streptozocin-induced type 1 diabetes in laboratory rats, restoration of altered levels of brain-derived neurotrophic factor, interleukin-6, and tumor necrosis factor-alpha in plasma, as well as increased levels of BDNF in the brain, pancreas and liver (Bathina and Das 2021).

Serum BDNF and VEGFA as Biomarkers of Diabetes Complications

Despite the fact that the study of the role of neurotrophins in the pathogenesis of diabetes began with their study in diabetic neuropathy, the problem of neuroplasticity in the structure of neurological disorders in diabetes remains relevant to this day.

Damage to the nervous system occurs from the earliest stages of hyperglycemia and progresses steadily with the course of diabetes. First of all, autonomous thin C-fibers have been involved in process, which leads to dysregulation of adaptation processes in the body, disorders of the basic functions of internal organs, primarily the cardiovascular system, which leads to the development of deadly complications such as a fixed pulse, uncontrolled arterial hypertension, and painless myocardial ischemia (Tesfaye et al. 2010). Another common manifestation of damage to the nervous system is distal symmetric polyneuropathy (DPN) of upper and lower extremities. Damage of lower extremities is associated with the development of

diabetic foot syndrome (DFS). Any impairment to the nervous system is a difficult-to-treat condition, the restoration of nerve fibers occurs slowly and directly depends on the length of the nerve fiber and the coordinated work of the mechanisms of neuroplasticity (Feldman et al. 2019). Shematic representation of pathogenesis of diabetic polyneuropathy is shown in Fig. 5.

The variety of pathways for damage to the nervous system opens up many potential biomarkers. One of the key points of compensation of pathogenic damaging links is the work of neuroplasticity and its key players – growth factors as the main mechanisms of adaptation of neural structures to pathological conditions (Leinninger et al. 2004). Since the discovery of neurotrophic factors, ideas about the possibility of their use as biomarkers of diabetic polyneuropathy have been expressed.

In particular, growth factors have been studied in diabetic neuropathy since the 1990s. In early experimental models of mice with streptozocin-induced diabetes, an increase in BDNF and TrkB expression in damaged sural nerves was determined immunohistochemically, which correlated with the regenerative capacity of nerve fibers (Zochodne 1996; McMahon and Priestley 1995).

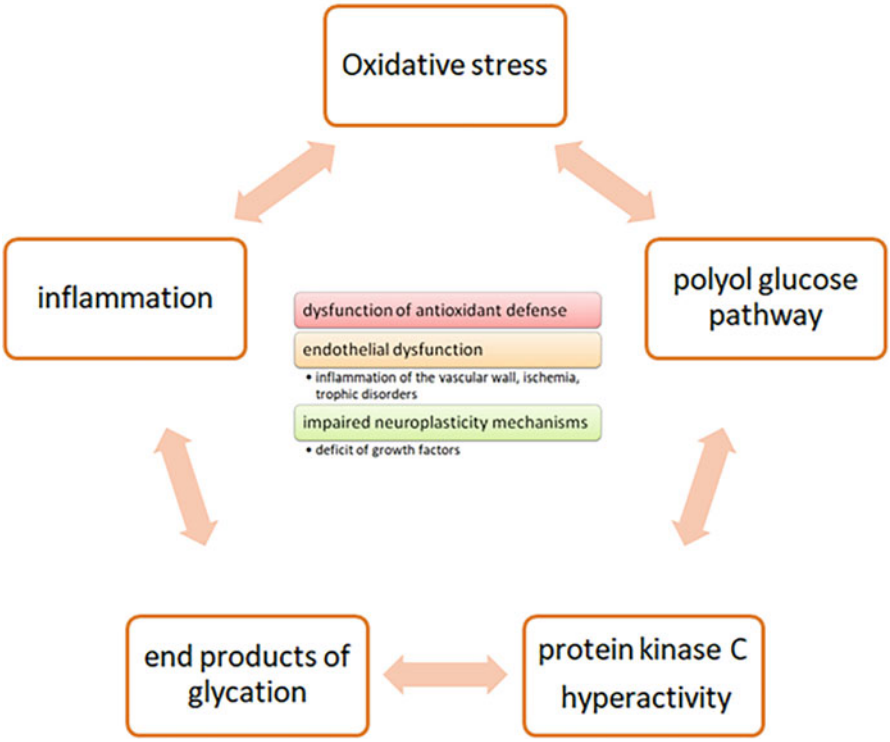

Fig. 5 Pathogenesis of diabetic polyneuropathy

In rat models of streptozocin-induced diabetes, an increase in the content of VEGF in the nerve fiber and the posterior spinal ganglia, as well as the dependence of its synthesis on insulin treatment, were demonstrated (Samii et al. 1999).

In an experimental model of ischemic polyneuropathy, gene therapy with vascular endothelial growth factor, carried out by direct transfer of VEGF DNA into ischemic muscle, prevents nerve conduction disturbances, in contrast to the comparison group with a similar degree of ischemia (Schratzberger et al. 2000). It was reported that vascular endothelial growth factor also stimulated migration and prevented ischemia-induced apoptosis of Schwann cells in which VEGFR2 receptors directly appeared. Later, the same group of researchers conducted a similar design study in laboratory mice with streptozocin-induced diabetes. Twelve weeks after the VEGF gene transfer, nerve conduction disturbance was completely restored both in respect of unmyelinated sensory fibers and motor myelinated according to electroneuromyography results. In addition, blood flow and the number of endoneurial vessels were restored to levels observed in non-diabetic animals. These data suggest that, in addition to restoring blood flow in ischemic nerve fibers, VEGF can directly contribute to the survival of peripheral nerve fibers. These functional characteristics make it possible to consider VEGF as a direct neurotrophic factor in diabetic polyneuropathy.

In addition, the neurotrophic activity of vascular endothelial growth factor was confirmed in the study by Sondell M; in the experiment, VEGF stimulated axonal sprouting and increased cell survival and proliferation of Schwann cells in the peripheral nervous system (Sondell et al. 1999). However, some authors have doubt we can use the vascular endothelial growth factor as a biomarker of neural tissue damage in diabetes. Since vascular endothelial growth factor is involved in the pathogenesis of multiple complications in diabetes, therefore, increased expression of VEGF may be just an universal response to damage and cannot be considered specific for neurodegeneration (Veves and King 2001).

Nevertheless, the experimental study, conducted by Taylor SL, on exogenous administration of low doses of VEGF to diabetic laboratory mice deficient in VEGF and its cognate receptor in the hippocampus demonstrated restoration of factor expression and improved memory (Taylor et al. 2015). The results of the study allow us to judge the vascular endothelial factor as a potential neuroprotective factor in diabetes mellitus.

Study of Serum Concentrations of BDNF and VEGFA in Human Populations in Diabetic Polyneuropathy

Independent groups of Chinese and Russian scientists have proven the possibility of using serum levels of brain-derived neurotrophic factor as a diagnostic marked of DPN.

Thus, in the Sun Q study (Sun et al. 2018), serum levels of brain neurotrophin and nerve growth factor were studied in three groups: patients with diabetic polyneuropathy, a group of patients with type 2 diabetes without polyneuropathy, and a

healthy control group. The levels of the considered factors were significantly lower in the group of patients with DPN and correlated with the course of the disease, C-peptide, and the level of glycated hemoglobin.

Karakulova Yu.V. et al. studied serum concentration of BDNF and VEGFA at various stages of diabetic polyneuropathy: subclinical, stage of clinical manifestations and in diabetic foot syndrome, as well as in a group of patients with diabetes without clinical and electrophysiological signs of DPN and in a healthy control group (Karakulova and Filimonova 2021). Serum levels of BDNF and VEGFA in the group of patients with diabetes without DPN did not differ from the group of healthy controls. In other groups, differences in concentrations were obtained, for example, patients with subclinical stage of DPN had increased levels of BDNF compared to healthy controls, serum levels of patients with clinically pronounced DPN symptoms were also high and correlated with the severity of neuropathic pain, while in the group of patients with diabetic foot syndrome, an absolute deficiency of the both growth factors in serum was obtained, a demonstrated in Figs. 6 and 7, accordingly. These results were explained by the authors as the initial activation of compensatory processes of neuroplasticity with an increase in the expression of brain-derived neurotrophic factor and vascular endothelial growth factor in response to damage to peripheral nerve fibers, the subsequent role of the BDNF in the formation of central sensitization of neuropathic pain, and, at a later stage, complete

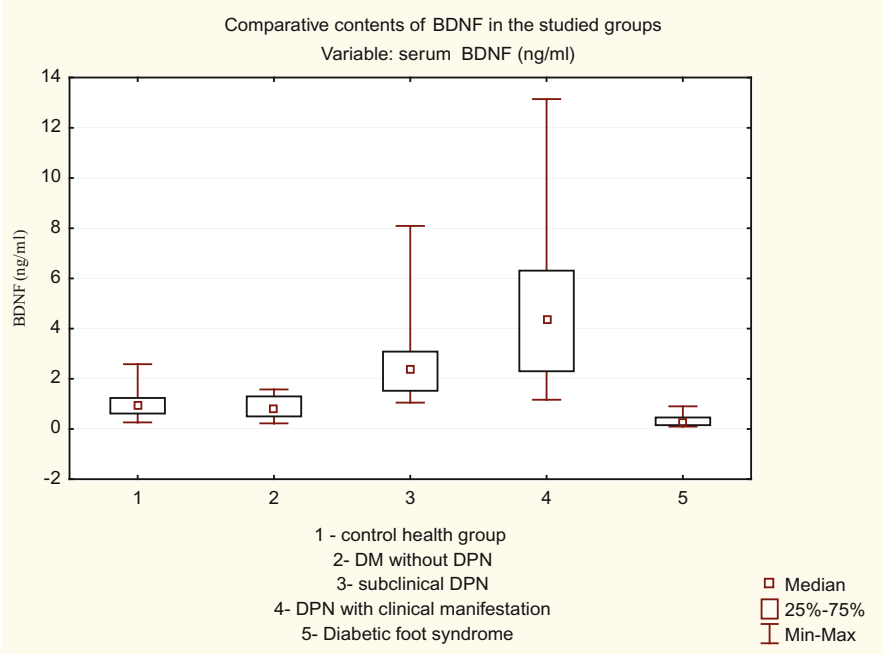

Fig. 6 Comparative contents of BDNF in the studied groups. (Images are used with permission of the authors)

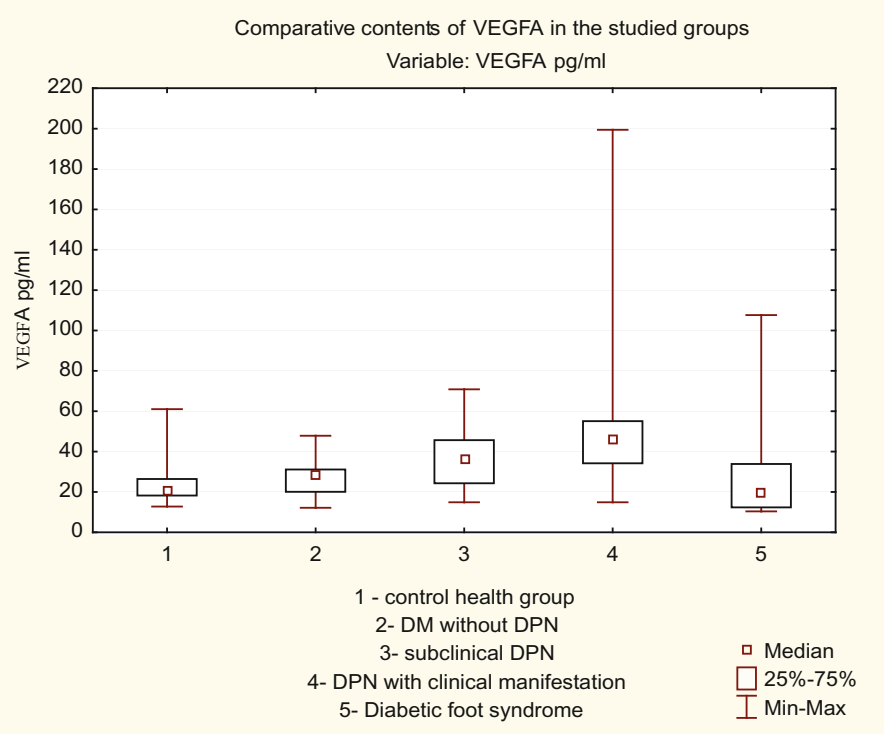

Fig. 7 Comparative contents of VEGFA in the studied groups. (Images are used with permission of the authors)

depletion of reserves of growth factors with decompensation of the process and pronounced clinical and electrophysiological parameters of DPN.

In recent studies, the dominant role in progression of diseases' pathogenesis is assigned to a defect in the receptor apparatus of growth factors. In particular, Karakulova Yu.V with coauthors (Karakulova and Filimonova 2021) studied the concentrations of highly specific receptors TrkB and vascular endothelial growth factor receptor (VEGFR2) along with the determination of serum concentrations of BDNF and VEGFA. There was the significant increase in TrkB in patients with DPN, regardless of the form, compared to those without DPN and in the healthy control group. However, no statistically significant differences were revealed among the various subgroups of DPN patients. Graphic representation of the concentrations is shown in Fig. 8. The serum level of VEGFR2 significantly increases only at the stage of clinical manifestation of DPN; in other subgroups, its concentration was comparable to that of healthy controls. Since the TrkB receptor is intramembrane and does not have active forms in the blood, its detection in serum can be considered a marker of nerve fiber damage, which was confirmed by the correlations obtained in the study – an increase in TrkB level may indicate an initial direct damage to the nerve fiber, which correlates with a decrease in the conduction rate along the nerve

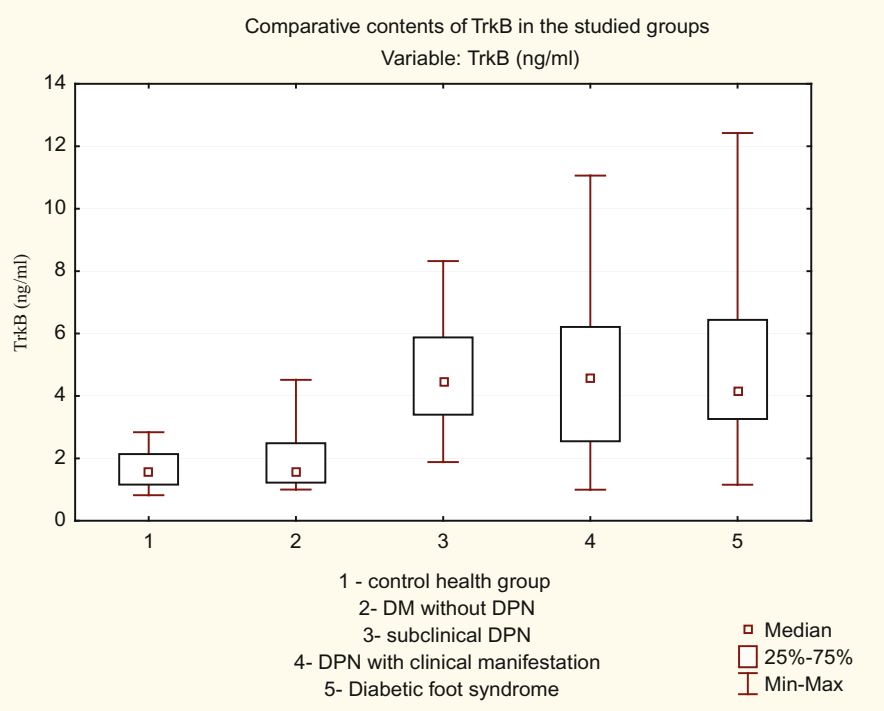

Fig. 8 Comparative contents of TrkB in the study. (Images are used with permission of the authors)

and an increase in distal latency according to the results of electroneuromyography of lower extremities. In addition, the authors also suggested using the level of TrkB as a predictor of the development of diabetic foot syndrome.

Further research will investigate how brain neurotrophin signaling overlaps with angiogenic factors, as well as identify vectors for targeting one of the factors with potentiation of the effects of the other as possible therapeutic targets for diabetic polyneuropathy.

Differences in Serum Levels of Neurotrophins in Diabetic Polyneuropathy in Type 1 and Type 2 Diabetes Mellitus

There is insufficient scientific evidence about differences in concentrations of BDNF and VEGF in diabetic neuropathy in type 1 and type 2 diabetes. There is a hypothesis according to which the pathogenesis of DPN in type 1 and type 2 diabetes differs so much that the authors propose to consider them as two different diseases (Callaghan et al. 2012). This hypothesis is supported by data on the absence of clinical and electroneuromyographic improvements in patients with type 2 diabetes and DPN, with one glycemic control, while such

monotherapy is sufficient to control clinical and electrophysiological changes in DPN with type 1 diabetes (Feldman et al. 2017). This issue requires additional in-depth study.

Modulation of Neuropathic Pain by BDNF in Diabetic Neuropathy

The role of BDNF and other neurotrophic factors in the generation of pain syndrome and positive sensory symptoms in diabetic polyneuropathy has been studied, but this theory has not received proper development in the future. BDNF expression was detected in presynaptic spinal neurons under the influence of pain impulses, as a result of which the neurotrophin is currently positioned as a mediator of central sensitization in neuropathic pain syndrome. Scientific opinions on the type of receptor involved in the formation and maintenance of neuropathic pain are currently divided. According to one point of view, transmission of the cerebral neurotrophin signal through nonspecific p75 promotes sensitization by releasing sphingosine-1-phosphate, modulating K + −ion channels, and increasing neuronal excitability (Obata et al. 2006). However, the authors acknowledge that the exact mechanisms of action of BDNF-p75 remain unclear. Since the expression of tyrosine kinase Fyn, which activates sodium channels and neuronal hyperexcitability, depends on the activation of both types of receptors, both trkB and p75, it is difficult to understand which pathway leads to sensitization (Hildebrand et al. 2016). Taken together, these results indicate that BDNF can enhance action potential through a variety of ion channels. The second point of view is based on the transmission of signals through the specific receptor TrkB. This theory is supported by the results of experimental studies, according to which mice lacking trkB exhibit a decreased response to pain and tactile stimuli (Cappoli et al. 2020).

Central Nervous System Disturbances in DM: The Role of Growth Factors

As for the brain damage in diabetes mellitus, only experimental data in animal models about a content of neurotrophins in the central nervous system are available at the moment. Studies show that the concentration of BDNF is reduced in the central nervous system of animals with modulated diabetes mellitus, and this deficiency can be compensated through aerobic exercise through endurance training (Eyileten et al. 2017). Some studies describe intrathecal injection of BDNF in animal model with T2DM, yet the number is limited and only based on neuropathic pain caused by diabetes. As for the human population, the measurement of growth factors in the central nervous system is technically impossible, and the concentrations of neurotrophins in the peripheral blood, according to most researchers, are not equivalent to those in the central nervous system (Rozanska et al. 2020).

Applications to Prognosis, Other Diseases, or Conditions

The neuroprotective effects of growth factors underlie the development of a strategy for controlling the signaling system of neurotrophic factors in treatment of diabetic polyneuropathy. Exogenous administration of BDNF in early neuropathy in rats with streptozotocin-induced diabetes provoked the significant improvement in nerve conduction. However, the use of neurotrophic therapy in the human population fell short of expectations. The first discovered neurotrophin, nerve growth factor (NGF), was tested first in a 3-phase clinical study of patients with DPN who received a 3-mg injection of recombinant NGF. The results showed no significant clinical improvement; changes in the patient's condition were only associated with local irritation at the injection site (Apfel et al. 1998). Further, a two-phase, double, placebo-controlled, randomized study was conducted in two experimental groups of people. The first received recombinant NGF at a dosage of 0.1 mg/kg and the second 0.3 mg/kg for 6 months, 3 times/week in the form of subcutaneous injections. Significant side effects were obtained in the form of paresthesias, hyperalgesia in the injection zone, and myalgia (Apfel 2002). The subsequent implementation of the third phase of this study, which consisted in the introduction of recombinant NGF in a lower dosage, allowed minimizing side effects; however, contrary to expectations, it did not demonstrate a therapeutic effect. Attempts have also been made to use a recombinant brain-derived neurotrophin for the treatment of diabetic polyneuropathy, but no visible improvement has been obtained (Wellmer et al. 2001). Cross-activation of p75-receptor, inflammatory reactions, high susceptibility of BDNF to proteolytic enzymes, and instability in the external environment determine the limitations of its therapeutic potential.

Despite the unsatisfactory results of the use of recombinant neurotrophins in diabetic polyneuropathy in humans, research in this area continues. Further studies are aimed at modulating the activity of specific receptors bypassing the action of growth factors by creating specific agonists based on neuropeptides. At present, encouraging results have been obtained with the use of TrkB agonists in the treatment of Alzheimer's, Huntington's, Parkinson's disease, traumatic brain injury, and even during physiological aging (Notaras and van den Buuse 2019).

Trials of VEGF genetic therapy in a treatment of diabetic foot are also promising. However, such studies are limited, firstly, by the mitogenic effect of VEGF and the possibility of developing proliferative processes, and secondly, by the inability to treat one vascular deposition without aggravating the other. Thus, for peripheral angiopathy and polyneuropathy, enhanced angiogenesis is required, but the question remains whether systemic treatment can aggravate proliferative retinopathy, and vice versa (Zubair and Ahmad 2019).

Several experimental studies in animals have shown the possibility of increasing the level of BDNF through aerobic physical activity. In the work of Tang and coauthors (Tang et al. 2017), the direct mechanism of this connection was studied in more detail: the study compared a group of rats with diabetes and a group of healthy rats in physical rest and the same groups that were subjected to physical exertion – climbing stairs and a treadmill. A group of diabetic rats without exercise

was characterized by a reduced level of BDNF and the cAMP-response element binding protein (CREB) gene in the hippocampus and decreased learning and memory ability compared with the normal control group. Groups of rats exposed to aerobic exercise showed higher levels of these parameters, as well as increased expression of the brain neurotrophin receptor in the hippocampus. Thus, the effect of physical activity on the learning ability of animals with diabetes was demonstrated, and this phenomenon could be associated with the activation of the BDNF/TrkB/CREB signaling pathway.

Clinical studies in a population of healthy people have demonstrated higher levels of serum granulocyte colony stimulating factor (G-CSF) in individuals actively engaged in physical activity and sports compared with healthy individuals leading a sedentary lifestyle. In addition, people with active lifestyles showed the best results in memorization and memory. However, no significant differences in BDNF concentration were obtained (Flöel et al. 2010).

In addition to the ability to enhance the production of endogenous BDNF, there is evidence of the possibility of activating its specific receptor TrkB without the participation of a ligand, that is, brain-derived neurotrophin. In particular, this effect can be achieved by a stimulation of G-protein coupled receptors, such as adenosine receptor 2A or dopamine receptor type 1 (Assaife-Lopes et al. 2010; Iwakura et al. 2008). Besides, there is evidence of autophosphorylation of the TrkB, but the exact mechanisms and trigger factors are still not clear (Middlemas et al. 1994). Thus, there are promising possibilities for controlling the receptor apparatus by modulating signals outside the presence of a growth factor. This scientific direction requires additional research.

Key Facts of Brain-Derived Neurotrophic Factor and Vascular Endothelial Growth Factor A: Biomarkers' Potential in Diabetes

- Recently increased interest in the development of novel biomarkers of diabetes mellitus and its complications is due to the high global prevalence and high mortality rate – according to the World Health Organization, 463 million people with diabetes were registered throughout the world in 2019.
- According to the IDF Diabetes Atlas 9th edition, 1 in 2 adults with diabetes is undiagnosed (232 million people)
- The sharply increased incidence of type 2 diabetes is due to the prevalence of urbanization, a sedentary lifestyle, malnutrition, obesity, and bad habits.
- At the time of diagnosis of diabetes mellitus, a person may already manifest severe labor-intensive complications that increase mortality. Early diagnosis would prevent the development of complications by taking preventive therapeutic measures.
- Numerous studies have proven the metabotropic role of the brain-derived neurotrophic factor, while the vasculoendotelial growth factor is capable of exhibiting neuroprotective activity and, along with BDNF, participates in glycemic control, inflammatory reactions, and metabolic disorders developing in diabetes.

Mini-Dictionary of Terms

Advanced glycation end products – proteins or lipids that undergo pathological reactions after irreversible binding with excess carbohydrates

Central sensitization – a pathological process in structures of central nervous system developing due to a damage of pain pathways and leading to the development of chronic neuropathic pain

Gluconeogenesis – a group of metabolic reactions of glucose formation from non-carbohydrate precursors

Insulin resistance – a dysfunction of insulin receptors with the inability to properly capture the ligand and ensure its functions – glucose utilization

Leptin – an active hormone-regulating eating behavior

Neuroplasticity – a property of nervous systems to change under an experience, as well as to restore damaged structures in response to external influences

Neuropathic pain – a type of pain syndrome that occurs directly in damage of various areas of sensory pathways of nervous system and characterized by specific sensations, usually described as shooting, burning, or tingling

Pancreatic beta cells – insulin-producing cells of the pancreas islet

p75 – low-affinity receptor for all neurotrophins, which provides the opposite role to high-affinity receptors, for example, p75 can trigger apoptosis

Streptozocin-induced diabetes – the most studied experimental model of diabetes, used in laboratory animals, by the administration of substances toxic for pancreas beta cells

Summary Points

Numerous experimental and clinical studies confirm the relationship of the altered amount of brain-derived neurotrophic factor with the development of insulin resistance, obesity, and inflammation in the context of diabetes mellitus. Brain neurotrophin is able to modulate neuropathic pain in the development of diabetic neuropathy. Deficiency of BDNF can underlie in the progression of irreversible damage to the structures of central and peripheral nervous systems. Experimental data on the participation of BDNF in the protection of pancreatic beta cells are promising. However, clinical studies in the human population so far show conflicting data and require additional research and meta-analyzes.

Vascular endothelial growth factor is a powerful angioprotective agent, its deficiency in diabetes mellitus provokes the development of endothelial dysfunction, micro- and macroangiopathic complications of the disease. In addition, VEGF is able to influence inflammatory cascades and metabolic control in diabetes mellitus. Considering the variety of functions, knowledge, and potential in relation to the nervous system, VEGFA was chosen as the object of further studying as the marker of diabetes mellitus and its neurological manifestations.

Thus, on the basis of various data, BDNF and VEGF can be classified as potential biomarkers in predicting the development of T2DM and may play a special role in

the treatment of this disorder and its complications. Peripheral measurements of BDNF and VEGFA in a blood provide a potential way to study the pathogenesis of the disease. Further researches are needed generating novel insight how to optimally modulate neurotrophins mediated signaling for discovery of novel therapeutics to improve diabetes and its complications.

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The Role of Glycated Albumin as a Biomarker of Glycemic Control in Diabetes and Chronic Kidney Disease

24

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Contents

Introduction 515
Albumin Discovery and Structure 515
Albumin Glycation Process 516
Diabetes Mellitus (DM) 518
Diabetes Mellitus and Glycation 518
The Use of Glycated Albumin in Monitoring Metabolic Control 520
Pitfalls of Conventional Measures of Glycemia 521
Fructosamine and Glycated Albumin 522
CKD and Diabetes 524
HbA1c for Glycemic Control in CKD 524
GA and Dialysis 526
Challenges and Pitfalls in the Measurement of Glycated Albumin 530
Conclusions, Limitations, and Recommendations 531
Applications to Prognosis and Other Diseases and Conditions 531
Key Facts of Glycated Albumin 532
Mini-Dictionary of Terms 532
Summary Points 532
References 533

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Abstract

Albumin is the most abundant protein found in plasma and constitutes approximately 60% of all proteins in the blood. This high molecular weight protein maintains the pH balance and osmotic pressure of blood. Albumin transports metabolic products and performs antioxidant functions. Glycated albumin (GA) is produced during the glycation of proteins and constitutes 80% of glycation reactions in plasma. GA has become useful in the monitoring of blood glucose levels and is often used with glycated hemoglobin measurements. The measurement of GA is favored in patients with hemoglobinopathies or those receiving dialysis. This chapter will discuss the advantages and disadvantages of GA use, including its role in diabetes control and in diabetes-associated complications.

Keywords

Glycated albumin · Diabetes · Chronic kidney disease · Dialysis · Glycated hemoglobin · Biomarkers · Fructosamine · Glycation products · Hyperglycemia

Abbreviations

ADA	American Diabetes Association
AGEs	Advanced glycation end products
BMI	Body mass index
CGM	Continuous glucose monitoring
CKD	Chronic kidney disease
CVD	Cardiovascular diseases
DKD	Diabetic kidney disease
DM	Diabetes mellitus
ESKD	End-stage kidney disease
FDA	Federal Drug Association
GA	Glycated albumin
GFR	Glomerular filtration rate
HbA1c	Glycated hemoglobin
HPLC	High-performance liquid chromatography
ICAM-1	Intracellular adhesion molecule 1
IDF	International Diabetes Federation
kDa	Kilodaltons
LMICs	Low-to-middle-income countries
OGTT	Oral glucose tolerance test
RBC	Red blood cell
RI	Reference interval
ROS	Reactive oxygen species
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VCAM-1	Vascular cell adhesion molecule 1

Introduction

Albumin is a high molecular weight protein composed of a single polypeptide chain. It is the main protein found in plasma and constitutes approximately 60% of all proteins in the blood. The concentration of albumin in the blood is roughly 35–50 g/L, with a half-life of 14–20 days (Anguizola et al. 2013; Danese et al. 2015). Albumin functions to maintain pH balance and the osmotic pressure of blood and acts as a transporter of metabolic products, including drugs and ions. Further, albumin is known to function in an antioxidant capacity (Ueda and Matsumoto 2015). Glycated albumin (GA) is the higher glycated portion of fructosamine, which is produced during the spontaneous, nonenzymatic glycation of proteins (Anguizola et al. 2013). GA is the main serum fructosamine and constitutes 80% of the total glycation reactions in plasma (Anguizola et al. 2013). In recent decades, GA has gained popularity as a method to monitor blood glucose levels, either as an alternative to or in conjunction with glycated hemoglobin (HbA1c). This chapter will discuss the characteristics, uses, benefits, and pitfalls of GA measurement regarding diabetes and associated complications.

Albumin Discovery and Structure

Albumin was one of the first proteins to be discovered and studied. Paracelsus is credited with the precipitation of serum albumin from urine as far back as the year 1500, and almost four centuries later, August Gürber successfully crystallized the protein in 1894 (Fig. 1) (Hamilton 2021). Ever since, knowledge about the structure of the protein and its various interactions with many molecules continues to grow, more than 600 years after its initial discovery. The total blood plasma protein content of healthy individuals is between 35 and 50 g/L (Rondeau and Bourdon 2011). While human blood plasma is composed of a wide range of proteins, albumin is its main constituent, accounting for 60% of the total protein. This makes albumin the most abundant protein in circulation, and its importance is demonstrated by its crucial role in maintaining the body's physiological homeostasis. It accomplishes this by performing many crucial tasks, including the maintenance of colloidal osmotic pressure in the vasculature (Danese et al. 2015).

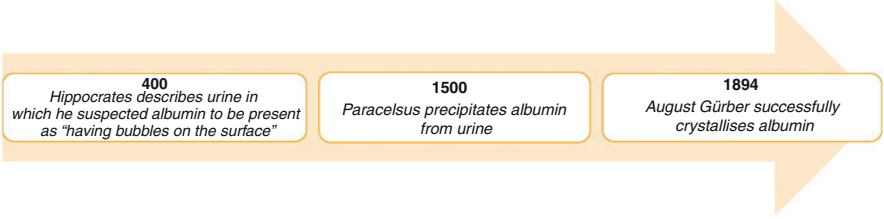

Fig. 1 Albumin history. Schematic showing the history of albumin from the fourth century to date

Over 30 years ago, the work of Carter and colleagues gave rise to the initial resolution of the three-dimensional structure of albumin (Carter et al. 1989). Albumin is a high molecular weight, globular protein of size 65–70 kDa, made up of a single polypeptide chain, with over 580 amino acid residues (mostly lysine and arginine). Albumin also comprises 35 lysine residues, which are involved in the stabilization of the molecule through the formation of 17 disulfide bridges (Carter et al. 1989). Overall, the structure of albumin is organized into three homologous domains (I, II, and III) connected in a helix (67% alpha-helices, 23% extended chains, and 10% beta-turns) (He and Carter 1992; Anguizola et al. 2013) to form a heart-shaped molecule, with each domain composed of two sub-domains (A and B) that have common structural motifs. Sub-domain A consists of six helices, while sub-domain B consists of four helices (Kragh-Hansen et al. 2006). Albumin's lysine and arginine residues, primarily lysine 525, increase susceptibility to glycation (Shaklai et al. 1984).

Albumin Glycation Process

Glycation, sometimes referred to as the Maillard reaction, is a nonenzymatic reaction in which the carbonyl group of a reducing sugar (glucose, galactose, fructose) or its breakdown products react with the free amino group of a protein (Anguizola et al. 2013; Singh et al. 2014). In the case of glycated albumin, the sugars bind to the free amino groups found on albumin, which are often arginine, lysine, or cysteine (Danese et al. 2015). The result is the reversible formation of a Schiff base product (Fig. 2) and, after Amadori rearrangement (carbohydrate modification), a more

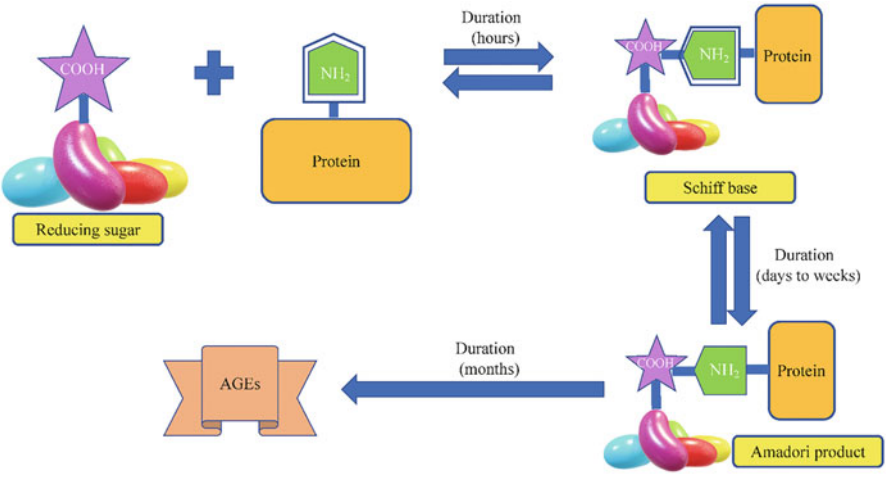

Fig. 2 The Maillard reaction. The process of glycation from its early stages during Amadori product formation to the advanced stages, with the formation of advanced glycation end products (AGEs)

stable ketoamine. These are products of the early stages of glycation. However, when these products are further modified through cleavage, oxidation, and rearrangement reactions, irreversible conjugates called advanced glycation end products (AGEs) are formed. Oxidation of adducts on glycated proteins or free amino acid residues leads to the formation of intermediate compounds, which can enhance cysteine residue modification or react with lysine or arginine, culminating in the formation of AGEs (Anguizola et al. 2013).

The structure of albumin enables the molecule to perform its various functions, including hormone, nutrient, and ion transport and pH and osmotic blood pressure maintenance (Freitas et al. 2017). Owing to its flexibility, albumin can alter its configuration to suit the molecule to which it is bound. However, when glycation occurs, this induces modifications to the protein's three-dimensional conformation and binding properties (Rondeau and Bourdon 2011), rendering it unable to effectively carry out its functions (Bourdon et al. 1999). For instance, the oxidation process that accompanies albumin glycation leads to a reduction in the antioxidant properties of albumin (Rondeau and Bourdon 2011).

Due to albumin's relatively longer half-life and its availability in greater quantities, it is highly susceptible to the glycation process (Rondeau and Bourdon 2011) and as a result has become an interesting biomarker target for short-term to intermediate monitoring of blood glucose control (Anguizola et al. 2013; Danese et al. 2015). As previously mentioned, one of albumin's main functions is the transport of different molecules to various sites in the body, which is made possible by the ligand-binding properties of albumin. Albumin can bind and transport drugs to their target sites. However, the glycation of albumin significantly alters the protein's tertiary structural conformation, subsequently affecting its ability to bind to several ligands (Shaklai et al. 1984). Clinically, the glycation of albumin influences the pharmacodynamics of drugs taken for various medical conditions, thereby influencing drug efficacy. This is because the glycation process may reduce albumin's binding affinity to the drugs, which was reported by Soudahome and colleagues, who found that glycated albumin's affinity for liraglutide (a drug with good efficiency in the management of type 2 diabetes) was reduced in diabetes patients, thus complicating the patient management process (Gajahi Soudahome et al. 2018).

Glycation is a major nonenzymatic alteration to which albumin is exposed throughout its life span, resulting in an inevitable impact on the body's metabolism (Rondeau and Bourdon 2011). For instance, GA may cause irreversible organ damage to the kidneys. This is because at higher levels, GA uptake is enhanced and is transported across the glomerular vasculature, where it is taken up by epithelial and specialized cells of the mesangium, and contributes to pro-oxidant molecule and type IV collagen formation in these cells, culminating in the initiation of kidney pathology (diabetic nephropathy) (Ziyadeh and Cohen 1993; Rondeau and Bourdon 2011). The pathological effects exerted by GA are also evident in cardiovascular diseases (CVDs), where it has been reported to promote platelet activation and aggregation, as well as the expression of intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), processes which are essential in the buildup of atherosclerotic plaques. In hyperglycemic conditions,

protein glycation leads to the development of AGEs, which accumulate inside cells and affect extra- and intracellular spaces. Due to their distinct ability to form cross-linkages with various molecules in the extracellular matrix basement membrane, AGEs contribute to micro- and macrovascular complications, as well as the atherosclerosis process (Goldin et al. 2006). Additionally, various complications of diabetes, including retinopathy and neuropathy, have been associated with GA (Nathan et al. 2014).

Diabetes Mellitus (DM)

Diabetes mellitus (DM) is a group of heterogeneous metabolic disorders characterized by the presence of hyperglycemia. This hyperglycemia may be attributed to insulin secretion impairment, defective insulin action, or both (Punthakee et al. 2018). Chronic hyperglycemia is associated with long-term microvascular complications affecting the eyes, kidneys, and nerves, as well as macrovascular complications, subsequently leading to increased risk of CVD development (Punthakee et al. 2018). Of the different types of DM, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the most common. T1DM accounts for approximately 10% of all diabetes cases, while T2DM accounts for the majority of cases (approximately 90%) (International Diabetes Federation 2019). DM is a major global burden, with approximately 463 million diabetes patients reported worldwide in 2019 (International Diabetes Federation 2019).

Previously only considered a public health crisis in high-income countries, recent years have demonstrated increased prevalence of DM in low-to-middle-income countries (LMICs) as well, with an estimated 79% of people living with diabetes residing in LMICs. The International Diabetes Federation (IDF) reported that the African region specifically may witness a 143% increase in the number of people diagnosed with DM by 2045. Furthermore, South Africa exhibited the highest age-adjusted prevalence of DM, the highest number of deaths, as well as the highest diabetes-related expenditure (23% of the total health budget), of all countries within the African region (Fig. 3) (International Diabetes Federation 2019).

Diabetes Mellitus and Glycation

DM is dependent on a combination of environmental factors, as well as genetic factors associated with impaired insulin secretion and insulin resistance (Kohei 2010). Most individuals with T2DM are elderly, with visceral obesity (linked to insulin resistance, as well as lack of exercise), and have a family history of DM. Furthermore, these individuals usually present with hypertension (Baynes 2015). In states of obesity and hyperglycemia, there are elevated levels of glycation (Nowotny et al. 2015). Although this process may occur under normal circumstances, protein glycation is accelerated in hyperglycemic states, as a result of elevated circulating glucose levels (Nowotny et al. 2015).

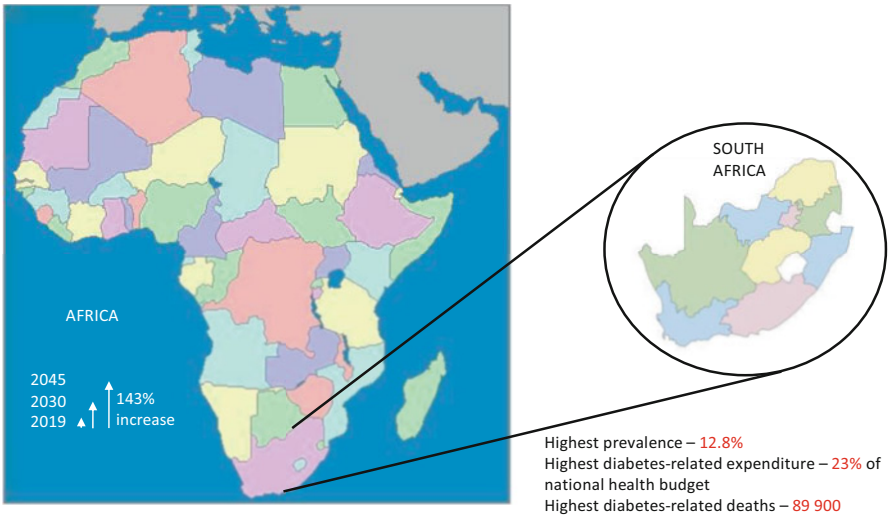

Fig. 3 Regional and national prevalence of type 2 diabetes mellitus. South Africa exhibits the highest prevalence, the highest number of diabetes-related deaths, and the highest diabetes-related expenditure of all countries within the African region

Glucose also has the ability to interact with, and bind to, various classes of proteins, ranging from blood proteins to tissue-specific proteins (Welsh et al. 2016). For instance, reactions with the amino groups of hemoglobin in red blood cells (RBCs) result in the formation of HbA1c (Little and Sacks 2009). Similar interactions occur with blood proteins, resulting in the subsequent formation of a group of stable ketoamines called fructosamines, which is the collective measure of all glycated proteins circulating in the blood (Danese et al. 2015). Although not widely used, the clinical relevance of fructosamine estimation is growing, with proposals motivating its use in short-term glycemic assessment, as well as studies revealing strong predictive capabilities for diabetes development and microvascular complications (Parrinello and Selvin 2014; Selvin et al. 2014). GA measurement has also been proposed as an attractive alternative marker for glycemia, in comparison to conventional measures, with various research endeavors revealing associations between elevated GA levels and T2DM (Bourdon et al. 1999; Ribeiro et al. 2016; Dorcely et al. 2017).

Glycation of tissue-specific proteins also occurs, propelled by the increased concentrations of oxidizing agents, as well as reactive oxygen species (ROS), characteristic of hyperglycemic environments, yielding irreversible glyco-oxidation products, also known as AGEs (Peppa et al. 2003). AGEs are toxic compounds, contributing to the underlying inflammation and oxidative stress characteristic of hyperglycemic states, and the mechanisms involved include macrophage stimulation, promoting resultant cell death, cellular adhesion, and differentiation impairment, as well as stimulating immune cell migration to sites of inflammation (Nowotny et al. 2015). Increasing evidence suggests that AGEs are involved in

mechanisms promoting diabetic complications, such as nephropathy, retinopathy, and neuropathy, and monitoring AGE accumulation may yield predictive capabilities for the development and progression of diabetes-associated complications (Peppa et al. 2003; Nowotny et al. 2015; Chume et al. 2019).

The Use of Glycated Albumin in Monitoring Metabolic Control

The glycation of proteins is more pronounced in hyperglycemic conditions relative to normoglycemia (Rondeau and Bourdon 2011). Once albumin is glycated, it becomes detrimental to the homeostatic balance of the body. The strong involvement of GA in the development of diabetes-related complications and its contribution to the development of cardiovascular diseases have been previously reported (Rondeau and Bourdon 2011). While HbA1c is a good indicator of chronic hyperglycemia over a 4-month period and is routinely used to diagnose and monitor diabetes patients, its use does not always provide an accurate blood glucose control picture in individuals with rapidly changing blood glucose concentrations and in patient groups whose RBC turnover is altered (Chume et al. 2019).

A study showed that, relative to GA, HbA1c may not be as reliable a marker for monitoring diabetes patients with advanced kidney disease, because these patients have low RBC counts (indicating anemia). Thus, while glucose may be abundant, there may not be enough hemoglobin to bind. In addition, the RBCs of these patients may not live long enough to bind with glucose to form HbA1c (Inaba et al. 2007). A study by Nathan et al. showed that, relative to HbA1c, GA was a superior indicator of poor glycemic control among diabetes patients on dialysis (Nathan et al. 2014). Over time, GA may prove to be worthy of further investigation as a biomarker for blood glucose control monitoring for the following reasons:

- Being an extracellular protein, albumin reacts with glucose approximately ten times more than hemoglobin does (Arasteh et al. 2014).
- GA has a half-life of 21 days and can be used as an indicator of the short-term glycemic picture.
- It correlates well with complications of diabetes, as is the case with HbA1c.
- GA is not affected by hemolytic processes and/or hemoglobinopathies, as is the case with HbA1c (Chume et al. 2019).
- Unlike with conventional blood glucose testing (fasting blood glucose test), a fasting blood sample is not required for GA measurement. While patients are encouraged to fast before a blood sample is collected, their compliance cannot always be guaranteed. As such, a test that removes that possible discrepancy warrants further investigation.

Overall, the measurement of GA is a potentially useful method that may fill the void left when HbA1c and self-blood glucose measurement are not viable options due to the patient group under consideration. While studies show the impressive performance of GA as a diagnostic marker for diabetes (Chume et al. 2019), it is

important that instead of being touted as a replacement for HbA1c, GA should serve as a complementary test to HbA1c, so as to further clarify the glycemic picture and advise clinical steps with respect to patient management and/or treatment efficacy. To this effect, Chume et al. showed that although HbA1c and GA were associated with microvascular complications, the relationship strengthened when the glycemic indicators were analyzed in combination (Chume et al. 2019). Currently, the global implementation of GA as a sole biomarker for the diagnosis and monitoring of diabetes, though promising, is a distant reality. However, evidence shows that progress is being made, particularly in Japan, where a group from the country developed an accurate, automated enzymatic GA measurement assay, which is now being used in Japanese clinical settings (Kouzuma et al. 2002). The wide use of GA in screening for diabetes is certainly being promoted on the East Asian island, with various studies in Japanese populations showing evidence that supports the need to consider GA for screening and diagnostic purposes in diabetes (Yoshiuchi et al. 2008; Furusyo et al. 2011; Ikezaki et al. 2015). Despite this work, various challenges still need to be overcome if GA is to be universally accepted as an equal, if not superior, to HbA1c in blood glucose monitoring.

Pitfalls of Conventional Measures of Glycemia

Current methods of glycemic screening include assessing fasting blood glucose and performing the oral glucose tolerance test (OGTT), the latter considered the gold standard for diagnosing T2DM. However, the OGTT is considered cumbersome and lengthy, taking at least 2 h to perform (Stolk et al. 1995). Extensive pre-examination preparation is needed prior to conducting the OGTT, such as a suitable diet within 3 days of the test, as well as overnight fasting, the night before. Patients have reported poor tolerance, with common symptoms experienced, such as nausea and hampered gastric emptying.

Measuring HbA1c has been supported for routine use in glycemic monitoring by the American Diabetes Association (ADA) since 1988 (American Diabetes Association 1989). Concentrations of HbA1c are dependent on the concentration of circulating glucose in the bloodstream. Furthermore, HbA1c concentration is affected by the life span of RBCs, with the life span averaging approximately 120 days. As such, assessing HbA1c provides information on an individual's glycemic picture, spanning 8–12 weeks prior to the test (Hirst et al. 2014). A diagnosis of type 2 diabetes may be defined by an HbA1c reading $\geq$6.5%, whereas prediabetes is defined by a reading of 5.7–6.4% (Inaba et al. 2007; Arasteh et al. 2014). Concerns have arisen regarding the accuracy of HbA1c, with mounting evidence suggesting that results may be influenced by several factors, such as age, ethnicity, and hemoglobin abnormalities, ultimately affecting the reliability of the test (Kouzuma et al. 2002; Furusyo et al. 2011). A study reported an increase in HbA1c levels of approximately 0.1% for every 10-year increase in age, in normotolerant individuals, while Hispanic individuals exhibited inherently higher HbA1c levels (0.12%) in comparison to non-Hispanic Caucasians with normal

glucose tolerance (Davidson and Schriger 2010). Similar differences have been reported in African Americans compared to non-Hispanic Caucasians, alluding that HbA1c overestimates glucose concentration in African Americans (Kirk et al. 2006). A 2011 study conducted in South Africa determined that a lower cutoff of 6.1% was more sensitive in mixed ancestry individuals, in contrast to the globally accepted cutoff of 6.5%, further demonstrating ethnic variations in HbA1c (Zemlin et al. 2011). Hemoglobin variants, such as hemoglobin C, S, E, and D, are commonly identified variants contributing to interferences in HbA1c measurement. Of these variants, hemoglobin S is highly prevalent among Africans, measuring 20–40% in certain African ethnic groups (Stolk et al. 1995; Florkowski 2013).

Fructosamine and Glycated Albumin

In light of the concerns regarding the use of HbA1c as an accurate measure of glycemia, the hunt for alternative blood-based markers has led to the acknowledgment of fructosamine and GA, as either potential replacement markers or complementary markers, to be used in conjunction with currently used glycemic indices, such as HbA1c (Welsh et al. 2016). HbA1c presents a reflection of long-term glucose metabolism, over a period of 8–12 weeks, whereas measured fructosamine, which includes GA, provides a short-term reflection of mean glucose concentration. Circulating blood proteins, such as albumin, exhibit higher rates of glycation in comparison to their intracellular hemoglobin counterparts (Fig. 4), which is due to the direct exposure of these proteins to glucose, thereby suggesting that GA levels may represent a more accurate glycemic portrait, in relation to HbA1c (Freitas et al. 2017). A study conducted by Takahashi and colleagues cross-sectionally examined the relationship between GA and HbA1c in type 2 diabetes patients to assess the changes in GA and HbA1c levels in individuals following intensive insulin therapy

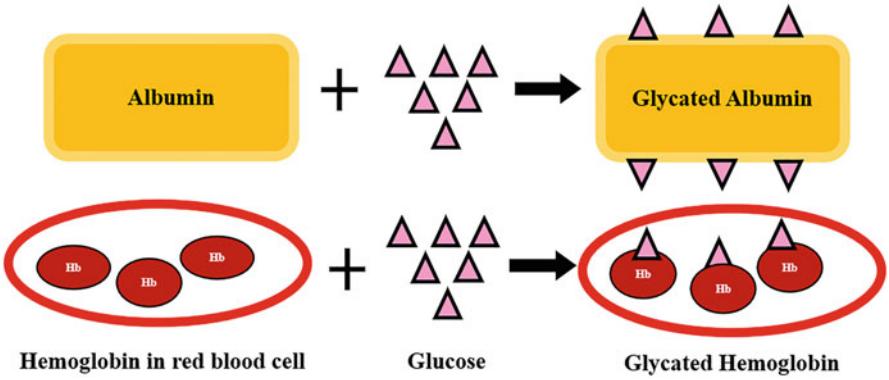

Fig. 4 Schematic showing the glycation of albumin and hemoglobin. Circulating albumin is more susceptible to glycation in comparison to intracellular hemoglobin since albumin experiences greater contact with glucose in circulation during hyperglycemic conditions

(Takahashi et al. 2007). Their observations were weak overall but significantly correlated between both markers. However, GA demonstrated a more rapid reduction, in comparison to HbA1c, in participants on insulin therapy. They ultimately motivated for the use of GA in monitoring short-term glycemic changes (Takahashi et al. 2007). As previously mentioned, a key determinant of HbA1c efficiency is the presence of hemoglobinopathies (Klonoff 2020). Contrastingly, reports have illustrated that GA is advantageous in that its measurement is unaffected by anemias, or abnormal hemoglobin (Furusyo and Hayashi 2013). An investigation aimed at assessing the relationships between indices of iron metabolism with both HbA1c and GA levels, in normal glucotolerant individuals with iron deficiency anemia, iron-deficient states, and normal iron states. Significant inverse relationships were determined between these markers and HbA1c, as well as significant differences in HbA1c levels between those with anemia and iron-deficient states, compared to those with normal iron states. No significant differences were observed in GA levels between the subgroups, signifying no influence on the overall accuracy of GA determination in glucose control (Koga et al. 2010).

Studies have also illustrated the superiority of glycemic monitoring using GA, compared to HbA1c, in patients presenting with diabetic complications (American Diabetes Association 2014; Selvin et al. 2014). Selvin et al. aimed to compare the performance of GA and fructosamine, versus HbA1c, in the identification of incident diabetes, as well as common diabetes-associated complications (Selvin et al. 2014). During the study, some participants eventually developed diabetes, and researchers observed that GA, fructosamine, and HbA1c demonstrated similar significant associations with risk of incident diabetes. In participants already diagnosed with diabetes, GA, fructosamine, and HbA1c levels demonstrated similar associations with risk of developing complications, such as retinopathy and chronic kidney disease (CKD). The investigators therefore proposed the incorporation of GA and fructosamine as additional markers for glycemic assessment (Selvin et al. 2014).

Circulating GA can be affected by disease states involving increased protein loss, such as nephrotic syndrome, as well as states of hampered protein synthesis, such as liver cirrhosis; thus, in these instances, it is not advisable to solely rely on GA measurement as an indicator of glycemic control (Bloomgarden et al. 2008; Danese et al. 2015). Additionally, due to the absence of a standardized assay for routine use in laboratory detection of GA, the reference intervals (RIs) are dependent on the detection method used; however, there is a mutual agreement among all detection methods that GA concentration is increased two- to fivefold in diabetes patients versus normotolerant individuals (Freitas et al. 2017). Ethnic variations in GA levels have also been reported between African and Caucasian Americans, with higher mean GA in African Americans versus Caucasian Americans (Kohzuma et al. 2011). Irrespectively, the overall benefits of measured GA justify its recognition and implementation in current strategies, particularly in financially constrained settings, such as Africa. Due to limited resources, there is a need for cost-effective testing methods, and GA has the advantage of being an economical alternative to expensive techniques, such as HbA1c testing (Danese et al. 2015). Studies evaluating the effectiveness of GA in African settings are lacking. A study headed by Zemlin

et al. evaluated the discriminatory power of GA in comparison to HbA1c, in distinguishing prediabetes and type 2 diabetes in South Africa. Sample-specific optimal thresholds were determined for both markers, and conclusions established an overall poor performance of GA in distinguishing either prediabetes or diabetes (Zemlin et al. 2019). A more recent study, also conducted in South Africa, determined otherwise, promoting the use of GA as a measure of dysglycemia (Peer et al. 2021). Taken altogether, there is corroborative evidence recommending GA as a prospective alternative, or adjunct in glycemic monitoring. With further studies backing this, as well as determining corrective measures for known influencing factors, these results may ultimately reinforce its inclusion in diagnostic strategies for dysglycemia, improving health outcomes worldwide.

CKD and Diabetes

CKD is a global public health concern affecting approximately 9.1% of the adult population worldwide (Bikbov et al. 2020). It is associated with increased risk of end-stage kidney disease (ESKD), CVD-related complications, and premature death (Bansal et al. 2017; Bikbov et al. 2020). Diabetes mellitus is a leading cause of CKD, accounting for approximately 40–50% of ESKD cases (Burrows et al. 2017). Studies have shown that diabetes-related death is often attributed to the development of kidney disease-related complications (Afkarian et al. 2013). Apart from being a cause of CKD, DM may also occur due to established CKD, which may exacerbate loss of kidney function, promoting progression toward ESKD. This is particularly concerning regarding the expected increase in DM prevalence globally, as this will increase ESKD incidence and subsequently increase the number of people requiring renal replacement therapy, thereby causing an economic strain on healthcare systems, particularly in developing regions, such as Africa and Asia (Liyanage et al. 2015). Therefore, it is essential to prevent the progression of DM to long-term microvascular complications, such as CKD, in order to reduce the enormous burden of ESKD and CVDs. Clinical evidence demonstrated that intensive blood glucose control may lead to good clinical outcomes in diabetes patients. Two landmark clinical trial prospective studies, the Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS), reported that intensive blood glucose control, as reflected by lower HbA1c and plasma glucose, has prevented or delayed the progression to diabetic nephropathy, diabetic retinopathy, and CVD outcomes in T1DM and T2DM, respectively (UKPDS Group 1998; Nathan et al. 2014). The evidence from these trials supported HbA1c as a widely used gold standard marker for assessing long-term glycemic control in clinical practice (UKPDS Group 1998; Nathan et al. 2014).

HbA1c for Glycemic Control in CKD

HbA1c is a product of nonenzymatic binding of an aldehyde group of glucose to the N-terminal valine amino acid of the hemoglobin beta chain (Jeppsson et al. 2002). It was discovered in the late 1960s in a study investigating novel hemoglobin variants

in blood, which identified that an "unusual hemoglobin," now called HbA1c, was increased in diabetes patients (Rahbar et al. 1969). HbA1c has several advantages, which makes it a preferable option; however, HbA1c also has several drawbacks that may limit its use in clinical settings. It has poor diagnostic performance in pregnant women and certain racial groups and is affected by several factors, such as age, hemoglobinopathies, hypertriglyceridemia, alcohol consumption, and certain anemias (World Health Organization 2011; Sacks et al. 2011; Speeckaert et al. 2014). Moreover, the clinical utility of HbA1c may be affected in the presence of certain disease conditions, such as CKD (World Health Organization 2011).

CKD, particularly at advanced stages, affects the life span of erythrocytes, thereby affecting the glycation of hemoglobin independent of plasma glucose. These factors can reduce or elevate the levels of HbA1c, thereby overestimating or underestimating glycemic control in these individuals. Erythrocyte life span and production are reduced in CKD, with 20–50% reductions observed in individuals on hemodialysis, resulting from high rates of erythrocyte lysis and loss of blood during the procedure (Vos et al. 2012). This reduction will in turn lead to falsely reduced HbA1c levels and overestimation of glycemic control. Frequent blood transfusions and treatment with iron supplements or erythropoietin-stimulating agents may lead to reduced HbA1c levels, due to enhanced erythropoiesis, which increases the proportion of young erythrocytes with reduced rates of glycation compared to older erythrocytes (Ng et al. 2010; Vos et al. 2012).

Anemia resulting from iron and/or vitamin B deficiency is a common feature in CKD; it is associated with an increased proportion of old erythrocytes, which subsequently increases the levels of HbA1c independent of glycemic effects, thereby underestimating glycemic control (Ng et al. 2010). The high levels of uremia and metabolic acidosis in CKD are associated with increased formation of carbamylated hemoglobin, which cannot be distinguished from HbA1c and thus interferes with HbA1c measurements when using certain analytical methods, such as high-performance liquid chromatography (HPLC), thereby falsely elevating HbA1c levels (Speeckaert et al. 2014). Moreover, studies have shown that the use of HbA1c for glycemic monitoring in diabetes patients with advanced CKD or on dialysis may be harmful, as it may result in an increased risk of hypoglycemia requiring hospitalization (Hodge et al. 2017). This may be due to reduced insulin clearance by the kidneys, resulting in prolonged insulin function, or by impaired kidney gluconeogenesis and other hypoglycemic agents. As a result of these factors, HbA1c values might not reflect the variability in glucose levels or the associated risk of complications. Therefore, interpretation of HbA1c levels in the presence of CKD should be performed with caution to avoid under/overestimation of glycemic control status.

Although clinical evidence suggest that establishing lower HbA1c levels by intensive glycemic control can prevent or delay the development of microvascular complications, reduce the risk of ESKD (Wong et al. 2016), and reduce the risk of morbidity and mortality in diabetic individuals on dialysis (Lee et al. 2016), other trials have shown that intensive glycemic control was associated with increased risk of CVD-related death and all-cause mortality in diabetes patients with CKD (early stages) when compared to those with normal kidney function (ACCORD Study Group 2008). Moreover, others found that intensive glycemic control had no

beneficial effect in preventing or delaying cardiovascular complications in patients with both diabetes and CKD (Patel et al. 2008). Despite the doubt surrounding the use of HbA1c in CKD, the ADA and Federal Drug Association (FDA) still recommend the use of HbA1c as a conventional marker of long-term glycemic control with HbA1c target value of <7% to prevent or delay progression to micro- and macrovascular complications in diabetes patients with or without CKD (American Diabetes Association 2016). Continuous glucose monitoring (CGM) has recently been recommended as an alternative marker for glycemic control monitoring in cases where HbA1c levels are uncertain, such as in the presence of CKD (Navaneethan et al. 2021). However, CGM is costly for clinical routine use and cumbersome to perform in individuals on dialysis (Navaneethan et al. 2021).

Other studies have investigated glycated proteins, primarily GA, as an alternative marker for the assessment of glycemic control in patients receiving dialysis or those with diabetes or advanced CKD (Table 1). Vos and colleagues assessed the markers of glycemic control in diabetes patients with CKD stage 4 or 5 and found that GA reflected glycemic control more accurately than fructosamine in diabetes patients with advanced CKD, whereas HbA1c showed poor correlation with mean blood glucose concentration (Vos et al. 2012). Similarly, Bellia et al. (2019) assessed the capability of HbA1c and GA to evaluate long-term glycemic control in diabetes patients with advanced CKD stage 4 or 5 with severe anemia and found that GA was the best marker (Bellia et al. 2019).

GA and Dialysis

Dialysis is the process of removing wastes and surplus water from the blood. This process is generally performed on patients with compromised kidney function. Hemodialysis uses a machine or artificial kidney, involves blood vessel access via the arm, and is often performed for patients without residual renal function. Peritoneal dialysis involves the use of the peritoneal membrane as a filter and is generally recommended for younger patients (Vadakedath and Kandi 2017). Hemodialysis replaces the functions of the kidney, but only to a certain extent, via diffusion and ultrafiltration (Vadakedath and Kandi 2017). CKD occurs over months to years and may progress to ESKD. These patients then require toxin removal, as kidney functions have decreased to ineffective levels. Prior to dialysis treatment, kidney function must be assessed to determine functionality. CKD can be diagnosed by assessing kidney function through the measurement of blood urea nitrogen or serum creatinine, a protein degradation byproduct, which estimates the glomerular filtration rate (GFR) (Vadakedath and Kandi 2017). Dialysis is typically recommended when the GFR falls below 15 mL/min/1.73 m^2. During dialysis, wastes and excess water are removed using a dialyzer and external filter containing a semi-permeable membrane. A counter-current flow gradient is then created to separate wastes, with blood flowing in one direction and dialyzer fluid flowing in the opposite direction. Solute particles diffuse across the membrane, and waste products (urea and creatinine) diffuse down the concentration gradient into the dialysate. Hemodialysis requires

Table 1 Observational studies assessing alternative markers for glycemic control monitoring in patients with diabetes or advanced CKD or receiving dialysis

Author and year	Country	Study cohort	Assay	Outcome
Inaba et al. (2007)	Japan	1731 individuals (828 hemodialysis patients, 538 hemodialysis patients with type 2 DM and 365 patients with type 2 DM and normal kidney function)	Casual plasma glucose, HbA1c and GA	GA was a better marker for assessment of glycemic control in hemodialysis patients with diabetes, while HbA1c underestimated glycemic control in this population
Vos et al. (2012)	New Zealand	Diabetes patients with CKD stage 4 and 5 or on dialysis	HbA1c, GA, and fructosamine	GA reflected glycemic control more accurately than fructosamine and HbA1c in diabetes patients on dialysis
Bellia et al. (2019)	Italy	81 CKD patients at stage 4 or 5, patients with moderate or severe anemia	GA, HbA1c, and fasting plasma glucose	GA was a better marker for evaluating glycemic control in diabetes patients with severe anemia, whereas HbA1c showed no association with fasting plasma glucose in these patients
Hanai et al. (2020)	Japan	841 chronic hemodialysis patients with diabetes, followed up for 3.1 years	HbA1c and GA	GA was a better predictor of all-cause and atherosclerotic cardiovascular disease mortality when compared to HbA1c in this population
Peacock et al. (2008)	USA	258 hemodialysis patients with DM and 49 diabetes patients with normal kidney function	HbA1c, CGM, and GA	GA reflects glycemic control more accurately in hemodialysis patients, while HbA1c underestimated glycemic control status
Hayashi et al. (2016)	Japan	41 type 2 DM patients on hemodialysis and 56 type 2 DM patients with normal kidney function	CGM, HbA1c, and GA	HbA1c was a superior marker for glycemic control monitoring in diabetes individuals on hemodialysis as compared to GA
Copur et al. (2021)	USA	1665 diabetes patients (without CKD (724), with moderate (464), severe (268) and very severe CKD (268))	HbA1c, GA, and fructosamine	GA or fructosamine showed no additional advantage over HbA1c for glycemic monitoring in diabetes patients with severe CKD

surgical access to large blood vessels and poses several additional health risks to patients, such as risk of infection and further inflammation (Murea et al. 2019).

Chronic inflammation is known to disrupt kidney function and promote accumulation of toxins, leading to ESKD. Dialysis may assist the kidney in regaining functionality by removing these toxins (Vadakedath and Kandi 2017). However, inflammation contributes to oxidative stress in dialysis patients. The dialysis membrane can become permeable to granulocytes, as it is subjected to interaction with IgG and complement components, initiating an immunological response. Granulocytes then promote ROS release. The kidney then activates macrophages and glomerular cells, resulting in further free radical production, which can eventually lead to multi-organ failure and death. The excessive production of ROS subsequently affects DM and CVDs, requiring increased intervention and therapeutic options for patients (Vadakedath and Kandi 2017). Many DM patients undergo dialysis due to the development of DKD, a microvascular complication of DM, which can lead to ESKD. HbA1c measurement is currently the gold standard for glycemic control and in monitoring the progression of DKD in DM patients. However, the use of HbA1c is flawed in patients with CKD (Copur et al. 2021), as they often present with anemia, vitamin or iron deficiencies, and reduced half-life of erythrocytes, which directly affect glycation of hemoglobin and may produce entirely unreliable results (Inaba et al. 2007). Glycated albumin has been recommended as an improved alternative to the use of HbA1c, especially for dialysis patients with DM. GA can be used as a biomarker to monitor and screen for DM and has been associated with the prediction of long-term disease outcomes (Freitas et al. 2017). HbA1c measurement is influenced by hemoglobin metabolism and is thus not recommended in certain clinical conditions due to interference in result interpretation (Sacks et al. 2002). However, fructosamine or GA levels are not affected by the concentration of other serum proteins and are specific to albumin glycation rates. HbA1c is further affected by hemolytic processes, while GA is not. This is of particular interest regarding patients with anemia or other hemoglobinopathies, as well as pregnant patients (Kim et al. 2010). DM or gestational DM patients would benefit from glycemic monitoring using GA, as opposed to HbA1c, since GA is not influenced by reduced iron levels toward the third trimester (Hashimoto et al. 2010). Therefore, GA is the preferred alternative to HbA1c during conditions that disrupt erythrocyte half-life or the structures and characteristics of hemoglobin (Fig. 5) (Freitas et al. 2017).

In patients with DM and CKD, HbA1c may not be entirely reliable, since patients may present with erythropoietin deficiency, develop anemia, or require blood transfusions, all of which will affect accurate HbA1c readings. During increased proteinuria, often seen in CKD, serum albumin levels are reduced and may produce inaccurate GA results. However, GA is measured as a ratio of glycated to total albumin, which may improve result accuracy during proteinuria (Danese et al. 2015). Thus, test choice is crucial for glycemic monitoring. GA sensitivity is considered better than that of HbA1c, since GA formation occurs over 2–4 weeks, while HbA1c formation occurs across the life span of erythrocytes (Koga 2014). This enhances GA sensitivity to alterations in blood glucose levels, which is

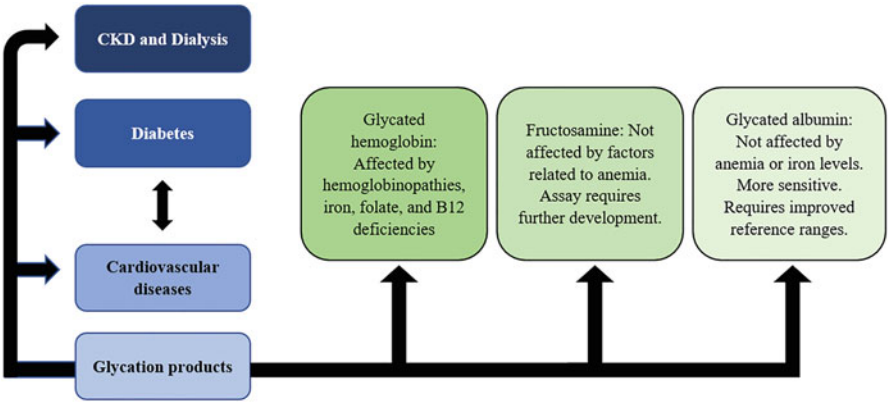

Fig. 5 Metabolic diseases and glycation products. Schematic indicating the relationships between chronic kidney disease, dialysis, diabetes, cardiovascular diseases, and glycation products (in relation to iron levels)

beneficial for monitoring and controlling DM treatment efficacy, such as drugs or insulin doses, as GA levels are influenced faster than HbA1c levels (Hsu et al. 2015).

HbA1c was assessed in hemodialysis patients, with GA and CGM used as references, and results showed that HbA1c measurements underestimated mean blood glucose levels in these patients (Peacock et al. 2008). Studies have found reduced erythrocyte life span in hemodialysis patients, which translates to reduced HbA1c measurements due to glycation occurring throughout the life span of erythrocytes (Vos et al. 2011), while the degree of GA indicates the mean blood glucose levels for the previous 3 weeks. Hematological factors that affect HbA1c readings do not affect that of GA; therefore, GA is considered more accurate and reliable for patients undergoing dialysis (Yajima et al. 2017). However, the hepatic turnover of albumin and albumin synthesis during proteinuria must be considered prior to interpretation of results (Okada et al. 2011). Peacock et al. conducted a study involving 307 diabetic subjects, 258 of which were undergoing dialysis and 49 without renal disease. These results indicated that dialysis significantly impacted HbA1c levels but did not impact the concentration of GA (Peacock et al. 2008). GA measurements accurately reflected glucose homeostasis in diabetic patients undergoing hemodialysis, while HbA1c levels underestimated glycemic control (Peacock et al. 2008). Several studies have agreed with the findings of Peacock et al., indicating that GA measurements are far superior in assessing glycemic control in diabetes patients receiving hemodialysis (Inaba et al. 2007; Gan et al. 2018).

In advanced glycation stages, oxidative and irreversible events produce stable, heterogenous compounds known as AGEs, which in turn increase oxidative stress and promote inflammatory cascades by activating NFκB (Freitas et al. 2017). Inflammatory kidney conditions are further exacerbated by these processes, which severely impact dialysis patients. Therefore, GA has been proposed as a biomarker of disease progression and disease outcomes, since GA is a precursor to AGEs and may increase oxidative stress, inflammation, and growth factor release (Cohen 2003;

Tan et al. 2007; Jun et al. 2018). GA may further serve as a potential treatment target for kidney disease in DM patients receiving dialysis. Furthermore, GA levels can also predict all-cause mortality in these patients. Research has shown that increased GA levels are better predictors of cardiovascular disease events and all-cause and cardiovascular mortality compared to HbA1c (Copur et al. 2021).

Taken together, research has clearly demonstrated the potential that GA measurement holds to aid in glycemic control for DM patients, especially those undergoing dialysis. GA may further be used to monitor treatment efficacy and disease progression, as well as predict the risks of future events. The role of GA should be investigated further at various CKD stages and observed in patients with DM and CKD at all stages of the disease. Further research is required involving dialysis patients across varying ethnicities and geographic locations.

Challenges and Pitfalls in the Measurement of Glycated Albumin

The vast differences in the methods used to measure GA present the possibility of measuring different glycation sites. This lack of standardization of GA determination methods presents a challenge, as inter-study findings may be difficult to compare if different methods were used to determine GA levels. Besides these methods being labor-intensive, making them difficult to adopt for routine use, they are also not available in most laboratories, further precluding their adoption for routine use in the laboratory measurement of GA (Furusyo and Hayashi 2013).

The early detection of a sustained increased in blood glucose is important, as corrective measures can be timeously implemented and complications due to chronic hyperglycemia can be avoided. In contrast to HbA1c, the short half-life of GA makes it a suitable biomarker for this purpose. In addition, it is not affected by RBC survival and hemoglobin metabolism disorders. However, in individuals with diseases like hypothyroidism, where the protein turnover is low, the measurement of GA as a glycemic control indicator may produce misleading results as the degree of glycation is influenced by low protein levels and may not mirror blood glucose concentrations (Furusyo and Hayashi 2013).

Previously, the use of GA as a biomarker of hyperglycemia was hampered by the lack of clearly defined cutoff values and RIs, as large-scale clinical trials to that effect had yet to be conducted (George and Erasmus 2018). However, more recently, more community-centric work has been conducted to mitigate this shortcoming. Using an enzymatic method to measure GA levels in healthy individuals in South Africa, Matsha and colleagues reported a RI of 10.7–15.2% for GA, noting the confounding effects of body mass index (BMI), sex, and ethnicity on the RI values (Matsha et al. 2019). Interestingly, these findings were echoed in a similar study conducted in the USA, where a RI of 10.1–15.1% was reported, citing BMI, sex, and race as confounding factors (Selvin et al. 2018). However, because of the population-centric nature of these RIs for GA, their applicability for clinical use remains a challenge.

Conclusions, Limitations, and Recommendations

In summary, glycated albumin has many advantages for glycemic monitoring, either as an alternative to or used in conjunction with HbA1c measurements. GA can be used to measure mean glycemia and evaluate glycemic variations, with greater sensitivity and reliability than HbA1c. GA exhibits rapid production and turnover for short-term and transient glucose monitoring, as well as treatment monitoring (Freitas et al. 2017), and GA measurement is recommended for hemodialysis patients. Interestingly, ethnic diversity plays a role in the successful use of GA in glycemic monitoring (Selvin et al. 2011). Further research is required to determine factors that interfere with GA values and to what extent. With the development of new, more standardized tests, the use of GA as a measure of glycemic control is becoming more appealing, especially in certain patients where the use of HbA1c is inaccurate and unreliable, such as patients that are pregnant, undergoing dialysis, or presenting with hemoglobinopathies. GA tests are becoming more affordable and require fewer specialized laboratory equipment. The methodology, kits, and protocols used for GA measurements should be investigated further to streamline and standardize testing procedures. Additionally, standardized, internationally accepted RIs and cutoff values are required to promote the use of GA measurement in diabetes.

Applications to Prognosis and Other Diseases and Conditions

Herein, we review the functions and uses of glycated albumin, including advantages and disadvantages of its use in patients with diabetes. We compare the differences between glycated albumin and glycated hemoglobin and their uses in glycemic control in patients with diabetes, and kidney and cardiovascular diseases. We discuss the limitations and advantages associated with using glycated albumin measurements while considering the influence of hemoglobinopathies, iron deficiency, and pregnancy. We further explore the use of glycated albumin as a biomarker to monitor glycemic control and optimize treatment options for patients with diabetes and other associated diseases, including chronic kidney disease, end-stage kidney disease, micro- and macrovascular illnesses, and cardiovascular complications. The use of glycated albumin is preferred in patients with hemoglobinopathies and those receiving dialysis, as the results are reliable and more accurate compared to the use of glycated hemoglobin alone for glycemic monitoring (Peacock et al. 2008; Ribeiro et al. 2016). Conditions that disrupt albumin metabolism, such as thyroid disorders, kidney disease, proteinuria, and liver cirrhosis, influence glycated albumin results. Further, age, obesity, triglyceride levels, inflammatory conditions, and smoking may affect GA levels (Danese et al. 2015). Further research is required to completely verify and standardize this method of glycemic control.

Key Facts of Glycated Albumin

- GA is formed as a result of glucose binding to albumin during elevated blood glucose circulation.
- GA has been proposed to replace or complement conventional testing measures for diabetes.
- With a half-life of 21 days, GA can be used as an indicator of short-term glycemic control, crucial for risk assessment and implementation of interventions.
- Benefits of its measure have also been observed in diabetes-associated complications, such as CKD, particularly in persons on dialysis.
- Despite growing promise, the lack of standardization is a hindrance for routine use of GA for screening and monitoring purposes.

Mini-Dictionary of Terms

- *Glycation:* nonenzymatic attachment of a carbohydrate to other biological molecules, such as proteins or lipids
- *Advanced glycation end products:* biomolecules (lipids, proteins) that become glycated due to long-term exposure to reducing sugars
- *Pharmacodynamics:* the effects of a drug on the body
- *Hemoglobinopathies:* diseases affecting red blood cells
- *Hemodialysis:* the artificial removal of wastes from the blood via an artificial kidney, in patients with kidney failure

Summary Points

- Albumin is an important component of blood and functions in various body processes that are essential for life.
- States of increased circulating glucose, such as diabetes, promote reactions between sugar and albumin, subsequently producing GA.
- Measuring GA has been touted as more efficient than HbA1c in short-term glycemic monitoring due to its faster turnover, and it remains uninfluenced by factors that affect HbA1c accuracy.
- GA has proven efficiency in glycemic monitoring in patients with end-stage kidney disease and patients receiving dialysis.
- Currently, the determination of reference intervals for GA hinders its clinical applicability in blood glucose monitoring.
- HbA1c remains the more universally accepted biomarker, although the use of GA is gaining impetus, particularly in Japan.

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Part III

Genetic, Molecular, and Cellular Variables

Epigenetics and 5-Hydroxymethylcytosines as a Biomarker in Type 2 Diabetes

25

Chang Zeng and Wei Zhang

Contents

Introduction 543
Novel Epigenetic Modifications beyond DNA Methylation 544
Technical Advances Enable Discovery of Hydroxymethylation Biomarkers 546
TAB-Seq 546
oxBS-Seq 547
The 5hmC as Novel Epigenetic Biomarkers for T2D Complications 549
Vascular Complications 549
Diabetic Retinopathy 551
Diabetic Nephropathy 553
Conclusions 554
Applications to Prognosis 555
Applications to Other Diseases or Conditions 555
Mini-Dictionary of Terms 555
Key Facts of Diabetic Complications 556
Summary Points 556
References 556

Abstract

Type 2 diabetes (T2D) is a lifelong chronic condition that impairs the body's normal function of regulating and using sugar (glucose) for energy. Continuous high blood sugar levels will eventually damage vital organs and lead to complications in various systems such as the circulatory, immune, and nervous systems. Complications associated with T2D affect quality of life and are the leading causes of death, thus representing a major public health burden. Considering the complex nature of T2D and complication development, exploiting epigenetic

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modifications holds the promise of detecting powerful biomarkers for the early detection of complications and providing potential targets for therapeutic intervention. Impressively, advances in epigenetic profiling during the past 20 years have allowed biomarker discovery targeting not only 5-methylcytosines (5mC), the most investigated epigenetic system to date, but also novel epigenetic modifications, particularly 5-hydroxymenthylcytosines (5hmC). Notably, the 5hmC are biochemically stable cytosine modifications that show a distinct genomic distribution and gene regulatory function from 5mC. Earlier studies have implicated 5hmC in complex diseases including cancer and cardiovascular diseases and explored their biomarker value using circulating cell-free DNA (cfDNA) derived from plasma. Considering the need for convenient epigenetic tools in clinical applications, in this chapter, we focus our discussion on the most recent advances in detecting 5hmC biomarkers for diabetic complications using a liquid biopsy- (i.e., cfDNA)-based approach. These technical breakthroughs lay the foundation of integrating novel epigenetic biomarkers in the care of T2D patients to improve clinical outcomes.

Keywords

Type 2 diabetes · Diabetic complication · Epigenetics · Biomarker · Methylation · Hydroxymethylation · Liquid biopsy · Cell-free DNA · Diabetic retinopathy · Diabetic nephropathy · Diabetic vascular diseases

Abbreviations

5caC	5-Carboxylcytosine
5fC	5-Formalcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
ALT	Alanine transaminase
AST	Aspartate transaminase
AUC	Area under the curve
BS-Seq	Bisulfite sequencing
BUN	Blood urea nitrogen
cfDNA	Cell-free DNA
DN	Diabetic nephropathy
DR	Diabetic retinopathy
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
HbA1c	Glycated hemoglobin
HDL-C	HDL cholesterol
IGF1R	Insulin-like growth factor 1 receptor
IL-10	Interleukin-10
LDL-C	LDL cholesterol
NGS	Next-generation sequencing
NPRD	Non-proliferative diabetic retinopathy

oxBS-Seq	Oxidative bisulfite sequencing
PDR	Proliferative diabetic retinopathy
T2D	Type 2 diabetes
TAB-Seq	Tet-assisted bisulfite sequencing

Introduction

Type 2 diabetes (T2D) is a lifelong chronic condition that features the impairment of regulation and use of sugar (glucose) for energy in the human body, with an estimated patient population of approximately 500 million, corresponding to 6–7% of the global population (Khan et al. 2020). Continuous high blood sugar levels, if not controlled, will eventually cause damages to vital organs and lead to complications in various systems such as the circulatory, immune, and nervous systems (Kolluru et al. 2012). T2D-associated complications may affect quality of life and cause death and disability, thus representing a major health burden (Khan et al. 2020). For example, for diabetic retinopathy (DR), a retinal vascular disorder caused by T2D and the leading cause of blindness, a previous epidemiological study indicated that more than four million US adults 40 years and older had DR and 1 of every 12 persons with T2D in this age group may have advanced, vision-threatening retinopathy (Kempen et al. 2004). In comparison, it is estimated that ~25% of diabetic patients will eventually develop diabetic nephropathy (DN), a serious kidney disease that is a leading cause of chronic kidney disease in Western societies (Gheith et al. 2015). As a matter of fact, T2D is responsible for ~30–40% of all end-stage renal disease cases in the United States (Umanath and Lewis 2018).

Strikingly, accompanying the trend of adoption of Westernized lifestyle in developing countries and the epidemic of obesity in developed countries, together with an aging population, it is expected that complications associated with T2D will continue to increase substantially as a major public health problem (Deshpande et al. 2008; Wu et al. 2014). Though existing laboratory tests and biomarkers such as proteinuria, GFR (glomerular filtration rate), albumin, and glycated hemoglobin (HbA1c) are useful in the diagnosis and monitoring of T2D-associated complications, there are currently no sensitive and specific biomarkers that can predict the early development of T2D-associated complications (Colhoun and Marcovecchio 2018; Galindo et al. 2020; Laakso 2019). Therefore, more powerful and convenient clinical tools that can exploit the complex nature of T2D and the development of complications (Fig. 1), i.e., the pathogenesis of T2D and complications is affected by a variety of genetic and non-genetic factors, are urgently needed (Forbes and Cooper 2013; Reddy and Natarajan 2011; Reddy et al. 2015; Sandholm and Groop 2018). Of particular interest are epigenetic biomarkers, e.g., cytosine modifications, that likely reflect earlier processes in the pathogenesis of T2D-associated complications when the genetics of T2D complications is difficult to define, considering the complexity and heterogeneity of these disorders (Gloyn and Drucker 2018; Kato and Natarajan 2014).

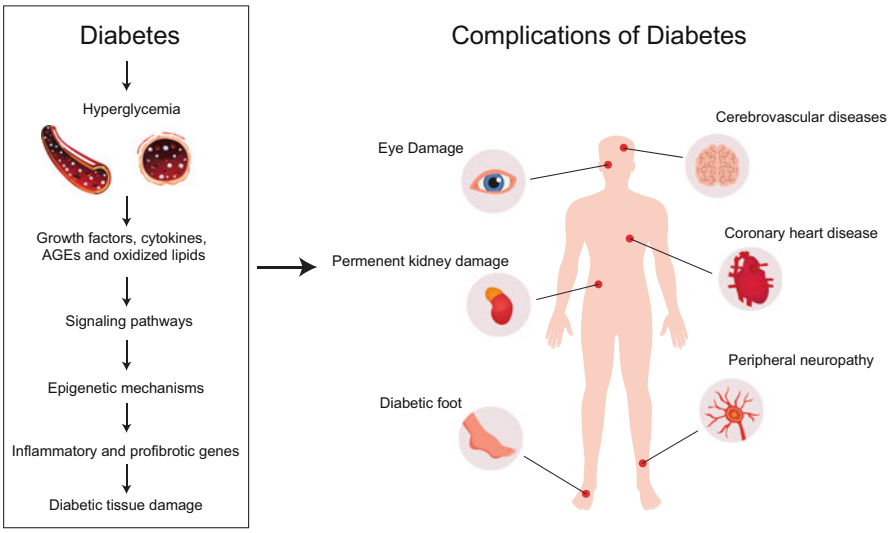

Fig. 1 The complex nature of T2D-associated complications. This figure shows the molecular mechanisms underlying hyperglycemia-induced tissue damage, which is the root cause of T2D-associated complications. High-glucose conditions induce the expression of growth factors, cytokines, advanced glycation end product (AGE), and oxidized products to promote diabetic tissue damage through signaling pathways and epigenetic regulations, which contribute to the progression of T2D-associated complications. For example, hyperglycemia can damage blood vessels and nerves in the peripheral nervous system, causing diseases including but not limited to retinopathy, nephropathy, and neuropathy

Notably, recent progress in liquid biopsy-based clinical tools for diagnosis and prognosis in other human complex diseases including cancer and cardiovascular disease (Heitzer et al. 2017; Rossi and Ignatiadis 2019; Zeng et al. 2019a), such as those based on novel epigenetic information, specifically 5-hydroxymethylcytosines (5hmC) in circulating cell-free DNA (cfDNA) derived from plasma (Figs. 2 and 3), has also opened up exciting opportunities for more effective management of T2D and early detection of T2D-associated complications (Han et al. 2021; Yang et al. 2019). Considering the convenience for future clinical implementation and the robust progress made by the research community during the past several years, in this chapter, we would like to introduce 5hmC and enabling technologies and then focus our discussion on exploiting this emerging epigenetic modification in cfDNA for the early detection of T2D-associated complications. We also provide perspectives for addressing the challenges toward clinical implementation in the field.

Novel Epigenetic Modifications beyond DNA Methylation

DNA methylation, i.e., the methylation of 5′-cytosines in CpG dinucleotides (5-methylcytosines or 5mC) in the human genome, has been the most investigated epigenetic modification (Fig. 2) (Laird 2003). The 5mC has been implicated

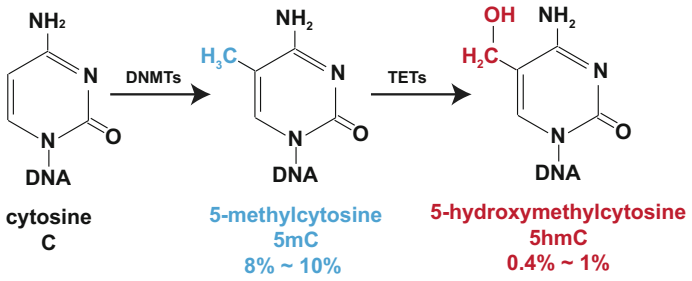

Fig. 2 5-Hydroxymethylcytosines are demethylated products of 5-methylcytosines. This figure shows the methylation of the DNA base cytosine to 5-methylcytosine and 5-hydroxymethylcytosine. Cytosine (C) can be methylated by the DNA methyltransferase family enzymes (DNMTs) to become 5-methylcytosine (5mC). Catalyzed by the Ten-Eleven Translocation (TET) family of enzymes, 5mC can be further demethylated to 5-hydroxymethylation (5hmC). Typically in the human genome, 8%–10% of cytosines are methylated, while only 0.4%–1% are hydroxymethylated, depending on tissues and cell states

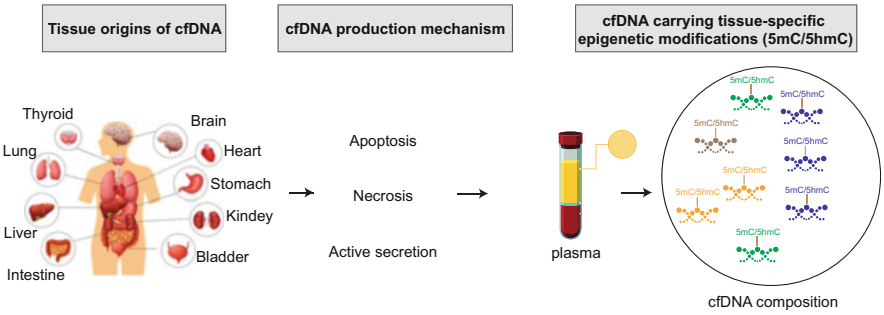

Fig. 3 Origins of circulating cell-free DNA (cfDNA). This figure shows the tissue origins and production mechanisms of circulating cfDNA. Circulating cfDNA is stable and fragmented cellular DNA released from cells into the bloodstream via mechanisms like apoptosis, necrosis, and active secretion. In healthy individuals, cfDNA is primarily derived from the apoptotic hematopoietic cells, whereas in diseased patients, cfDNA can come from affected tissues and their microenvironment, thus reflecting the epigenetic signatures of the affected tissues and microenvironments

in various biological processes and the pathogenesis of human complex diseases (Gokul and Khosla 2013; Laird 2003; Tost 2009). Conventional bisulfite conversion base microarray platforms or next-generation sequencing (NGS) approaches for 5mC profiling, however, do not distinguish 5mC from other less abundant modified cytosines (Barros-Silva et al. 2018). Therefore, previous studies on "5mC"/"DNA methylation" and complex diseases have limitations in that the findings are not specific to the types of modified cytosines profiled in their samples.

Notably, recent molecular biology indicated that the 5hmC, the second most abundant modified cytosines in the human genome, are biochemically stable epigenetic markers, not simply oxidized intermediate products of the 5mC (Fig. 2) (Branco

et al. 2011; Ito et al. 2011; Tahiliani et al. 2009). Interestingly, the genomic distributions of the 5hmC and 5mC are distinct from each other (Nestor et al. 2012; Zeng et al. 2019b). While the 5mC are known to be enriched in promoters and repress gene expression, the 5hmC are more likely to be located in gene bodies and enhancer markers, thus marking active expression (Han et al. 2016; Song et al. 2012; Zeng et al. 2019b). In addition, a recent analysis of 19 human tissues from 10 organs by Cui et al. shows that the 5hmC modifications are highly tissue specific and associated with genes specifically expressed in different tissues, suggesting the potential of using the 5hmC modifications to reveal whole-body phenotypes, such as a systematic chronic condition like T2D (Cui et al. 2020). Compared to the 5mC, the 5hmC may represent 1–10% of modified cytosines, still offering sufficient interrogative sites in the human genome for biomarker discovery (Fig. 2) (Branco et al. 2011; Nestor et al. 2012; Tahiliani et al. 2009). In contrast, although there are other modified cytosines in the human genome, such as 5fC (5-formalcytosine), these rare modified cytosines are under the detection level of current profiling technologies for clinically feasible biospecimens (Fig. 2) (Branco et al. 2011; Ito et al. 2011). Therefore, to understand the epigenetic mechanisms of complex diseases and exploit epigenetic modifications for their biomarker potential, enabling technologies that can sensitively and robustly profile 5hmC are critical for their clinical implementation (Table 1).

Technical Advances Enable Discovery of Hydroxymethylation Biomarkers

During the past decade, several profiling strategies have been proposed to measure 5hmC modification levels such as the Tet-assisted bisulfite sequencing (TAB-Seq) and oxidative bisulfite sequencing (oxBS-Seq) approaches (Table 1) (Booth et al. 2013; Yu et al. 2012). These two protocols were developed to detect and quantify genome-wide 5hmC at nucleotide resolution through modified sodium bisulfite sequencing (BS-Seq).

TAB-Seq

In the TAB-Seq protocol, 5hmC is first protected by β-glucosyltransferase (β-GT)-mediated glucosylation, and the genomic DNA is then treated with a recombinant Tet enzyme followed by sodium bisulfite conversion and PCR amplification. During this process, 5mC is converted to 5caC (5-carboxylcytosine) first and then to uracil (which is read as thymine [T] during sequencing), whereas glycosylated 5hmC remains untouched and reads as C (Yu et al. 2012). Therefore, a direct measurement of 5hmC abundance is gained from a single TAB-Seq run, while, in contrast to TAB-Seq, 5hmC abundance can only be inferred from oxBS-Seq, which will be discussed further thereinafter (Table 1).

Table 1 Genome-wide profiling technologies for 5-hydroxymethylation. This table summarizes and compares different genome-wide profiling techniques for 5hmC

Technique	Approach	Platform	Advantages	Disadvantages
Whole-genome TAB-Seq (Yu et al. 2012) *(direct measurement)*	Bisulfite conversion-based	NGS	Whole-genome Single nucleotide resolution Absolute methylation value	High sequencing depth required Input >500 ng DNA Computationally expensive
Whole-genome oxBS-Seq (Booth et al. 2012; Booth et al. 2013) *(inference)*				
RRBS TAB-Seq (Hahn et al. 2015) *(direct measurement)*			Single nucleotide resolution Absolute methylation value ~60 million PE reads/sample	Lack of coverage in non-CpG island regions
RRBS oxBS-Seq (Skvortsova et al. 2017) *(inference)*				
TAB-Array (Nazor et al. 2014) (*direct measurement*)		Array	Single nucleotide resolution absolute methylation value cost-effective	Coverage in targeted regions input >500 ng DNA
oxBS-Array (Stewart et al. 2015) (*inference*)				
5hmC-Seal (Song et al. 2011) *(direct measurement)*	Pull-down	NGS	Input 5 ng DNA (1000 cells) Excellent genomic coverage High specificity for 5hmC ~30 million PE reads/sample	Lower resolution Subjected to enrichment bias

oxBS-Seq

In the oxBS-Seq protocol, 5hmC is selectively oxidized to 5fC with an oxidizing agent (Booth et al. 2013). After the subsequent sodium bisulfite treatment of the genomic DNA, 5fC (derived from 5hmC) and cytosine are converted to uracil and read as T, while 5mC remains unchanged and reads as C. Therefore, a direct measurement of 5mC abundance is gained from a single oxBS-Seq run. The abundance of 5hmC is then inferred by subtracting the 5mC abundance from the total 5hmC and 5mC abundance measured from BS-Seq (Table 1) (Booth et al. 2013).

Although the original protocols for the TBA-Seq and oxBS-Seq were for the NGS platforms, both approaches have been implemented in microarrays, e.g., by integrating with the Illumina Infinium arrays to provide the TAB-Array and oxBS-Array (e.g., oxBS-450 K) protocols (Table 1) (Nazor et al. 2014; Stewart et al. 2015). Previous studies using these technologies have explored 5hmC biomarker potential in various diseases such as pancreatic cancer using the TAB-Array and glioblastoma using the oxBS-Array (Table 1) (Johnson et al. 2016; Zeng et al. 2019b).

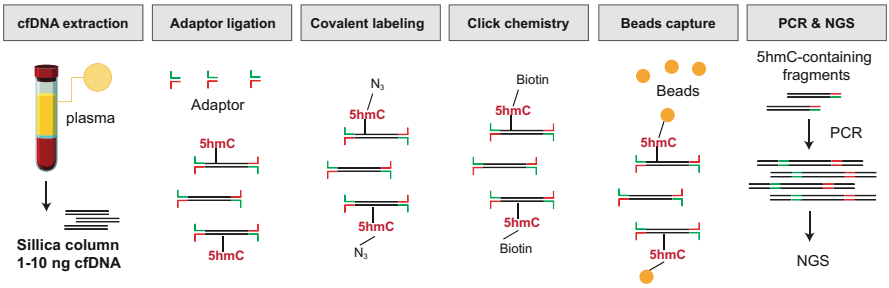

Fig. 4 The workflow of the 5hmC-Seal assay. This figure shows the general workflow of the 5hmC-Seal, a highly sensitive, chemical selective labeling technique to detect and quantify 5hmC in small amount of DNA. Briefly, purified cfDNA from plasma is ligated with Illumina sequencing adaptors. The 5hmC-containing cfDNA fragments are selectively labeled with a biotin group, followed by capturing on the avidin beads, PCR amplification, and next-generation sequencing (NGS)

Notably, these "gold standard" technologies (i.e., TAB-Seq and oxBS-Seq) require large amount of input DNA materials (e.g., 50–100 ng) that are often prohibitive for precious clinical biospecimens, particularly liquid biopsies like circulating cfDNA samples that feature only a few nanograms of DNA in clinically feasible amount of plasma (e.g., 5–10 mL) (Table 1) (Zeng et al. 2019a). Therefore, more sensitive profiling technologies for limited DNA input are necessary for exploring the biomarker potential of 5hmC in liquid biopsies and will be critical for future implementation in the clinic setting.

Specifically, the nano-hmC-Seal technology (i.e., 5hmC-Seal) was developed for highly sensitive 5hmC profiling taking advantage of the NGS (Table 1) (Fig. 4) (Han et al. 2016; Song et al. 2011). Briefly, cfDNA or fragmented genomic DNA is first repaired and ligated with adaptors. Next, the T4 bacteriophage enzyme β-glucosyltransferase (β-GT) is used to transfer an engineered glucose moiety containing an azide group to 5hmC in duplex DNA. A biotin tag is then installed onto the azide group using Huisgen cycloaddition ("Click") chemistry. Finally, 5hmC-containing DNA fragments with biotin tags are efficiently captured by avidin beads. A cDNA library is then constructed through PCR amplification and subjected to the NGS. Because of the sensitivity of chemical labeling, the 5hmC-Seal method is highly sensitive and reproducible (Fig. 4) (Han et al. 2016; Song et al. 2011).

The 5hmC-Seal method has been explored in cfDNA samples from healthy individuals and patients with various diseases including liver cancer, glioblastoma, lymphoma, colorectal cancer, multiple myeloma, pancreatic cancer, lung cancer, as well as cardiovascular disease (Cai et al. 2019, 2021; Chiu et al. 2019; Gao et al. 2019; Han et al. 2021; Li et al. 2017; Song et al. 2017; Yang et al. 2019; Zhang et al. 2018). Of particular interest are findings in diabetic complications using the 5hmC-Seal approach, which will be discussed in more detail in the following section.

The 5hmC as Novel Epigenetic Biomarkers for T2D Complications

Epigenetic contributors have been implicated in the development of diabetic complications, e.g., microvascular complications (Kumari et al. 2020; Reddy and Natarajan 2011). However, currently, they lack epigenetics-based tools that can sensitively and specifically detect these complications and monitor their progression. Taking advantage of the enabling technology, i.e., the 5hmC-Seal, several recent studies as discussed in this section have reported progress in exploring the 5hmC biomarkers for different T2D-associated complications using plasma-derived cfDNA samples (Han et al. 2021; Yang et al. 2019). The general study design (Fig. 5) is to compare genome-wide 5hmC profiles in cfDNA samples from cases (e.g., T2D patients with a particular complication) and controls (e.g., patients without the complication of interest). Together with differential analysis for more informative modified genomic features (e.g., gene bodies), machine learning algorithms are used to perform further variable selection and build models that target to distinguish cases from controls. Pathway and functional annotation analysis is used to implicate the identified 5hmc features in T2D-associated complications.

Vascular Complications

Study background – Because macrovascular and microvascular events associated with T2D are long-term complications and are the major causes for T2D-related disability and mortality, Yang et al. reasoned that a clinically convenient, non-invasive approach that can detect these complications in T2D patients would improve the patient's quality of life and contribute to the reduction of mortality and T2D-related healthcare burden through potential preventive interventions, if these complications are detected at stages that can still be intervened (Yang et al. 2019).

Methods – The 5hmC-Seal method was used to obtain genome-wide 5hmC profiles in a set of 62 patients with T2D, among whom 40 had vascular complications and 12 T2D patients had no complications (Yang et al. 2019). Specifically, among the 50 patients with vascular complications, 34 patients had so-called single vascular complications, i.e., these patients were diagnosed with only one type of vascular complication (macrovascular events, $n = 24$; microvascular events, $n = 10$), and 16 patients had multiple vascular complications, i.e., these patients were diagnosed with more than one type of vascular complications (Yang et al. 2019). These patients were randomly assigned into a training set and an independent testing set, and then the 5hmC-based models were trained in the training set of samples, followed by validation in the testing samples (Yang et al. 2019). The 5hmC-Seal data were summarized into gene bodies for differential modification analysis and further feature selection using the elastic net regularization, a flexible machine learning algorithm for genomic data and multivariable logistic regression models (Yang et al. 2019).

Results – In the training samples, Yang et al. identified 135 gene bodies with differentially modified 5hmC levels between patients with and without vascular

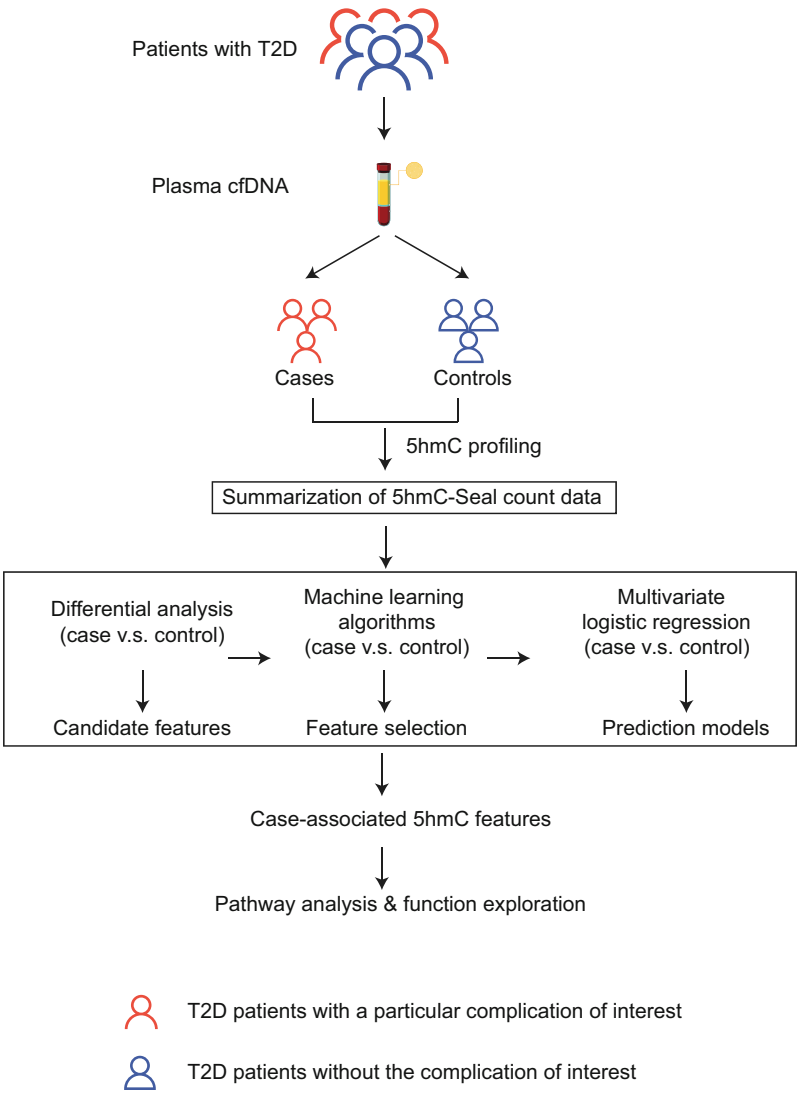

Fig. 5 The case-control study design to detect epigenetic biomarkers for T2D-associated complications. This figure shows the general case-control study design to detect cfDNA-derived epigenetic biomarkers for T2D-associated complications utilizing the 5hmC-Seal technique. Briefly, in order to develop a model for detecting cases (e.g., T2D patients with a particular complication of interest), differential analysis is used to identify a list of most informative candidate marker features; these candidate marker features are further selected to build a final panel of 5hmC marker features by applying statistical models or machine learning algorithms to distinguish cases from controls. Implicated genes or genomic features can be explored for their functional relevance through pathway or network analysis

complications (Yang et al. 2019). In contrast, they identified 159 differentially modified gene bodies between those patients with single and multiple vascular complications (Yang et al. 2019). Machine learning-based feature selection indicated a final panel of 16 genes that showed distinguishing capacity between patients with vascular complications, regardless of micro- or macrovascular events, and those with no complications (Yang et al. 2019). Between patients with single and multiple vascular events, Yang et al. also identified a panel of 13 gene bodies that separated these patients with different complication burdens (Yang et al. 2019). Combining the 5hmC-based diagnostic models in cfDNA and various clinical variables validated the biomarker potential of 5hmC for T2D-associated vascular complications in independent samples (Yang et al. 2019). Specifically, in the testing set, the AUC (area under the curve) of the 5hmC-based diagnostic model significantly outperformed laboratory variables including HbA1c, ALT, AST, eGFR, BUN, LDL-C, and HDL-C (Yang et al. 2019). Yang et al. further performed multivariable analysis to show that the 5hmC-based diagnostic models were an independent indicator for distinguishing patients with vascular complications from those without (Yang et al. 2019). Using publicly available functional annotation databases, many differentially modified genes were shown to have functions relevant to T2D-associated vascular complications (Yang et al. 2019). For example, functional annotation analysis of the 135 differentially modified gene bodies associated with vascular complications indicated enrichment of such pathways as signaling by insulin-like growth factor 1 receptor (IGF1R) and interleukin-10 (IL-10), which are relevant to insulin resistance and inflammation, thus relevant to T2D-associated complications (Yang et al. 2019). In contrast, those differentially modified gene bodies associated with developing multiple complications were found to be enriched in such pathways as cell-cell junction, collagen formation, metabolism, and cellular responses to stress (Yang et al. 2019).

Conclusions – The genome-wide 5hmC profiles in cfDNA from patients with T2D-associated vascular complications and those T2D with no complications, after controlling potential confounders (e.g., diabetic duration, age), showed differential modifications that could be exploited as potential biomarkers for the early detection of vascular complications or as monitoring tools in T2D patients integrated with routine care, considering the convenience of a blood-based test and non-invasiveness. In addition, it is possible to further differentiate T2D-associated vascular complications with different features (e.g., single vs. multiple complication events) (Yang et al. 2019).

Diabetic Retinopathy

Study background – The previous study by Yang et al. did not distinguish different types of vascular complications (Yang et al. 2019). To further explore the 5hmC-based biomarkers for a specific vascular complication, Han et al. recently published a case-control study that aimed to explore the 5hmC biomarkers for diabetic

retinopathy (DR), a common microvascular complication in patients with T2D (Han et al. 2021). Mechanistically, DR is caused by damage to the small blood vessels, thus being a type of microvascular complication, of the light-sensitive tissue at the back of the retina in the eye (Han et al. 2021). DR is the leading cause of blindness and may severely affect the patient's quality of life and increase the burden of health and family care. Therefore, clinically convenient tools that can be integrated into routine care and monitoring of T2D patients could help improve the detection, prevention, and treatment of this severe complication. The primary goal of Han et al. was to profile 5hmC in cfDNA and compare the profiles between patients and controls to investigate whether differential 5hmC features could be detected to be associated with DR, thus providing the basis for future biomarker development in cfDNA for this complication (Han et al. 2021).

Methods – The 5hmC-Seal method was used to profile genome-wide 5hmC profiles in 70 cfDNA samples in total form 35 T2D patients with DR, including 27 patients with NPDR (non-proliferative DR) and 8 patients with PDR (proliferative DR), together with and 35 age-, gender-, and diabetic duration-matched T2D patients without DR or other apparent complications (e.g., cardiovascular diseases, nephropathy) (Han et al. 2021). Differentially modified 5hmC features (i.e., summarized in gene bodies) were identified between cases and controls using the multivariable logistic regression models (Han et al. 2021). A machine learning algorithm was used to evaluate whether a subset of the differentially modified 5hmC features could be used to generally represent DR, compared to controls (Han et al. 2021).

Results – Specifically, out of the whole-genome features they compared between case and controls, a three-gene signature comprised of *MESP1*, *LY6G6D*, and *LINC01556* associated with DR was detected using the elastic net regularization-based feature selection and the multivariable logistic regression models (Han et al. 2021). Notably, the 5hmC-based model for DR demonstrated high accuracy to distinguish T2D patients with DR from those T2D controls with no DR or other complications, after controlling covariates such as age, gender, and diabetic duration, showing an AUC of 91.4% with a 95% confidence interval of 84.3–98.5%, significantly outperforming various risk factors and clinical variables (e.g., fasting glucose, blood pressure, triglycerides, and total cholesterol), for which the AUC ranges were 52.7–72.8% (Han et al. 2021). Likely limited by the sample size that was dominant with NPDR, the 5hmC-based model for DR showed comparable performance between patients with PDR and NPDR, compared to controls (Han et al. 2021). Interestingly, Han et al. tested DR patients from Yang et al. in the previous study to evaluate the performance of their 5hmC-based model for DR. Specifically, in this external testing set (i.e., Yang et al.), their three-gene 5hmC-based model for DR detected five out of six DR patients and predicted seven out of eight non-DR patients with other microvascular complications (Han et al. 2021). Therefore, these findings suggested that the detected 5hmC-based model for DR by Han et al. potentially reflected specific epigenetic characteristics to DR only (Han et al. 2021). Since the majority of the study subjects in Han et al. were patients with NPDR, the 5hmC marker panel they detected represented the first study that linked 5hmC to early stage of DR, thus laying the foundation for future exploration of the implications of these emerging epigenetic

marks in the prevention of DR (Han et al. 2021). Interestingly, *LY6G6D*, one of the final model genes, indicated relatively higher distinguishing capacity for PDR compared to the other two model genes (Han et al. 2021). *LY6G6D*, a gene involved in filopodia functions of fibroblasts and hypoxia-related strain response, therefore, appeared to be implicated in PDR (Han et al. 2021).

Conclusions – Han et al. further demonstrated that the 5hmC-Seal method in cfDNA could reflect 5hmC alterations specific to DR, a specific microvascular complication common in T2D patients, laying the foundation for future biomarker development for this severe complication (Han et al. 2021). In addition, this study, although focusing on one specific microvascular complication, suggested the potential of using 5hmC-based biomarkers to separate different types of microvascular complications, considering that specific 5hmC features were identified to be associated with RD (Han et al. 2021). Finally, although limited by the sample size, this study provided promising results to future investigations that aim to distinguish non-proliferative early stage (i.e., NPDR) and the proliferative late stage (i.e., PDR), which is critical for the precise care of this complication (Han et al. 2021).

Diabetic Nephropathy

Study background – Like other vascular complications, long-term damages that developed in the kidney, i.e., diabetic nephropathy (DN), a specific microvascular complication, represent a severe complication in patients with T2D. As a matter of fact, DN has become the most frequent cause of end-stage renal disease in most countries (Alicic et al. 2017; Umanath and Lewis 2018). Prediction of DN development can ultimately improve the patient's quality of life and reduce the burden of health and family care. Therefore, clinically convenient, non-invasive biomarkers for DN are urgently needed to control this severe complication in T2D patients. In the 2020 Annual Meeting of the American Diabetes Association, Zeng et al. presented a study that aimed to explore the 5hmC biomarkers for DN in cfDNA (Zeng et al. 2020).

Method – Genome-wide 5hmC profiles in 66 cfDNA samples from a set of 12 T2D patients with DN, 29 T2D patients with non-DN complications (non-DN, e.g., retinopathy and macrovascular complications), 14 T2D controls with no complications (T2D), and 11 controls without T2D were obtained using the 5hmC-Seal method and the NGS as described in previous studies (Zeng et al. 2020). Pair-wise comparisons between patients with DN and non-DN patients were performed to identify differentially modified 5hmC features as summarized in gene bodies, followed by feature selection to build a 5hmC-based model for DN using machine learning-based feature selection (Zeng et al. 2020).

Results – A panel of 271 differentially modified genes were detected between T2D patients with no complications and DN (Zeng et al. 2020). Pathway and functional annotation analysis suggested that these differentially modified 5hmC features were involved in pathways relevant to the synthesis of bile acids and bile salts, fatty acid metabolism, mitochondrial gene expression, and ion transport (Zeng et al. 2020). In

addition, a four-gene 5hmC marker panel (*DNAH1*, *RAB11FIP4*, *POLRMT*, *RAB36*) was found to be differentially modified among T2D patients with no complications, T2D patients with DN, and T2D patients with non-DN complications (Zeng et al. 2020). Further analysis indicated that this four-gene signature showed capacity for differentiating DN from T2D controls with an AUC of 83% and 95% confidence interval of 66–100%, or from healthy individuals with an AUC of 83% and 95% confidence interval of 64–100%, or from all combined non-DN patients with an AUC of 88% and 95% confidence interval of 76–100% (Zeng et al. 2020).

Conclusions – In this small sample size pilot study, the 5hmC features in cfDNA were further explored for their biomarker potential in a very specific microvascular complication associated with T2D, i.e., DN. Preliminary findings by Zeng et al. demonstrated that specific 5hmC features differentially modified between DN and controls involving relevant pathways were likely to be detected from cfDNA samples (Zeng et al. 2020). The 5hmC profiles in cfDNA reflected epigenetic changes in T2D patients with DN indicated the potential to be a clinically convenient, non-invasive approach for monitoring the presence of DN in high-risk T2D patients (Zeng et al. 2020).

Conclusions

Previous studies as described above showcased the current progress made by the research community to take advantage of novel epigenetic technologies for biomarker discovery for T2D-associated complications. There are several limitations in these studies. Firstly, the sample size of the current studies is still small. A robust biomarker panel or model will need to be developed and confirmed in larger sample size studies in the future. Secondly, although several major T2D-associated complications have been explored in the previous studies, future investigations will be necessary to be expanded to other major complication types, e.g., the nervous system (Han et al. 2021; Yang et al. 2019; Zeng et al. 2020). Additionally, with the expansion to more complication types, future investigations will be able to evaluate whether the 5hmC-based biomarkers are complication type-specific. Thirdly, although the 5hmC-Seal method is a highly sensitive and robust technology for profiling genome-wide 5hmC profiles, its pull-down nature does not provide information at base resolution. Future advances in enabling technologies may likely achieve highly sensitive 5hmC measuring at the base resolution for clinically feasible amount of input materials. Finally, although this chapter was focused on 5hmC, future investigations of other genetic and epigenetic biomarkers may need to be integrated to provide a more comprehensive signature of T2D-associated complications, considering the complex nature of these conditions.

In conclusion, with the advances in enabling technologies and our improved understanding of the epigenetic mechanisms for T2D and its complications, the research community is making progress with the ultimate goal of developing novel

epigenetic tools that can be implemented in the clinical setting to improve the clinical care of patients with T2D and the patient's quality of life.

Applications to Prognosis

In this chapter, novel epigenetic biomarkers, specifically 5hmC in cfDNA for diabetic complications caused by T2D, have been reviewed (Han et al. 2021; Yang et al. 2019; Zeng et al. 2020). *Although the current progress has been made in the diagnostic value of these epigenetic biomarkers for diabetic complications, it is reasonable to hypothesize that 5hmC biomarkers in cfDNA would have prognostic value as well, considering the complex nature of clinical outcomes. As a matter of fact, a few pilot studies using the 5hmC-Seal technique and the NGS have suggested prognostic value of 5hmC in cfDNA in oncology* (Chiu et al. 2019). *The research community is expected to make breakthroughs in this field in the near future.*

Applications to Other Diseases or Conditions

In this chapter, several recent studies investigating 5hmC biomarkers in cfDNA for complications associated with T2D have been reviewed (Han et al. 2021; Yang et al. 2019; Zeng et al. 2020). *In particular, the 5hmC-Seal technology, together with the NGS, was used in these studies to profile genome-wide 5hmC profiles in cfDNA derived from plasma* (Li et al. 2017; Song et al. 2011). *Due to its non-invasiveness and technical performance (*e.g., *high sensitivity), this technology has the potential to be utilized in other biomarker discovery research and holds the promise as a clinically useful tool in diagnosis, prognosis, and surveillance* (Li et al. 2017). *Considering the critical roles of epigenetic modifications in both normal biological processes and disease pathogenesis, biomarker discovery targeting these molecular targets and future advances in enabling technologies will have impact on other diseases and conditions, including those diabetic complications that have not been investigated yet.*

Mini-Dictionary of Terms

- ***Complex disease****. A condition that is affected by multiple genetic and non-genetic factors.*
- ***Complication.*** *A complication is an unfavorable result of a disease or treatment.*
- ***5-Methylcytosine.*** *A methylated form of cytosine (*i.e., *modified cytosine) in the DNA and the most common cytosine modification.*

- ***5-Hydroxymethylcytosine.*** *A hydroxymethylated form of cytosine in the DNA, whose distribution and function are distinct from 5-methylcytosine, and the second most common cytosine modification.*
- ***Cell-free DNA.*** *DNA fragments in the bloodstream that are not encapsulated in a cell or organelle.*

Key Facts of Diabetic Complications

T2D is a chronic condition by which the human body loses normal functions of regulating and using sugar as energy.

T2D affects hundreds of millions of the global population.

Long-time high levels of blood sugar in uncontrolled T2D cause damages to the human body, leading to complications.

Common complications caused by T2D include kidney disease, nerve problems, heart disease and stroke, eye disease, foot problems, gum disease, and skin conditions.

Severer complications caused by T2D are the leading causes of disability and death.

Summary Points

- *Complications caused by T2D affect a significant proportion of global population, being the leading causes for disability and death.*
- *Complications associated with T2D are complex diseases that are caused by multiple genetic and non-genetic factors.*
- *Emerging epigenetic modifications, particularly 5hmC, have been implicated in gene regulation and disease pathogenesis.*
- *Technical advances have begun to allow biomarker discovery that exploits 5hmC in the human genome, especially in clinically convenient biospecimens (*e.g.*, cfDNA).*
- *Previous studies, though limited by, for example, the relatively small sample size, provided novel insights into epigenetic biomarkers for T2D-associated complications.*
- *Future investigations will need to further refine and validate the previous findings in larger populations.*

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Adult Stem Cells as a Biomarker in Diabetes 26

Scott Cohen and Sabyasachi Sen

Contents

Introduction 560
Why Adult Stem Cells 561
Effect of Exercise on Endothelial Progenitor Cells (EPCs) 562
Effects on EPC 562
Age Is Another Major Factor Behind Reduction of EPC Numbers 562
Effect of Novel Diabetes Medications on Endothelial Progenitor Cells (EPCs) 563
Effect of HIV Medication Regimens on Endothelial Progenitor Cells (EPCs) 565
Effects on Exercise on MSCs 566
Conclusion 568
Applications to Other Diseases or Conditions 568
Mini-dictionary of Terms 569
References 570

Abstract

Obesity, diabetes, and cardiovascular diseases (CVD) are increasing rapidly worldwide. It is important to know the effect of exercise, medications for chronic diseases such as diabetes, HIV, and obesity on adult stem cells. Adult stem cells play a major role in remodeling of the body and tissue regeneration but may have an important role as a biomarker. In this review we will focus mainly on two adult stem/progenitor cells such as endothelial progenitor cells (EPCs) and mesenchymal stromal cells (MSCs). These two-adult precursor/stem cells are easily obtained from peripheral blood or adipose tissue depots, as the case may be and are precursors to endothelium and mesenchymal tissue (fat, bone, muscle, and

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cartilage), respectively. They both are key players in maintenance of cardiovascular homeostasis and can act as a useful biomarker in prediabetes, diabetes, and several disorders that impact metabolism.

Keywords

Endothelial progenitor cells · Mesenchymal stromal cells · Adult stem cells · Biomarkers · Diabetes · Obesity · Cardiovascular diseases

Abbreviations

ADMA	Asymmetric dimethyl arginine
ASCs	Adult stem cells
CAD	Coronary artery disease
CVD	Cardiovascular diseases
DM2	Type 2 diabetes
DPP	Diabetes prevention program
DPP-4	Dipeptidyl peptidase 4
EPCs	Endothelial progenitor cells
INSTI	Integrase inhibitors
MSCs	Mesenchymal stromal cells
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
SET	Supervised exercise training
SGLT2i	Sodium glucose channel inhibitors
TBI	Traumatic brain injury
VEGF	Vascular endothelial growth factor

Introduction

At present obesity, diabetes, hypertension, stroke, and coronary artery diseases (CAD) are rising dangerously, worldwide. According to NIDDK, one in three adults are obese and two in every three adults are overweight and obese (NIDDK 2014). WHO estimated 422 billion people are suffering from diabetes (World Health Organization 2017). The CDC also anticipates that one out of three adults suffer from hypertension. One out of every 20 deaths are due to stroke and one out of every four deaths are due to CAD in the USA (Center for disease control and prevention 2016; Center for disease control and prevention 2017a; Center for disease control and prevention 2017b). According to the CDC, 84 million Americans also have prediabetes (Center for disease control and prevention 2017c), a condition associated with increased risk of type 2 diabetes (DM2), cardiovascular disease (CVD) which includes hypertension, CAD and stroke. In addition to causes of mortality, these health conditions are associated with a large economic burden. The CDC estimates that the annual medical cost of obesity in the USA was $147 billion dollars (NIDDK 2014). The American Diabetes Association reported that it costs $245 billion dollars to treat diabetes and related complications in the USA (American Diabetes

Association 2013). According to the CDC, approximately $46 billion was used to treat hypertension, $34 billion for stroke and $200 billion for heart disease (Center for disease control and prevention 2016; Center for disease control and prevention 2017a; Center for disease control and prevention 2017b).

Diabetes medications and a non-pharmaceutical approach such as exercise has been shown to be effective in decreasing the cardiovascular risks associated with obesity and diabetes. The US Diabetes Prevention Program (DPP) validated that physical exercise significantly reduced the risk of developing CVD (The Diabetes Prevention Program Research Group 2002). According to the CDC, physical exercise not only reduced the risk of developing diabetes and CVD, but also helped to reduce body weight and even reduced the risk of developing certain cancers (Center for disease control and prevention 2015). In addition, exercise also strengthens bones and muscles, improves mental health and daily activity, and increases balance, thereby preventing falls. In combination with dietary changes, exercise increases life expectancy irrespective of age, ethnicity, body shape, or body mass (Center for disease control and prevention 2015).

Exercise and lifestyle changes have been shown to reduce chances of progressing from prediabetes to diabetes. It is however important to ascertain ideal biomarkers to detect the effect of both exercise and diabetes medications. The effect of exercise has been studied extensively using body composition parameters such as muscle mass, fat mass, weight, physical fitness (cardiovascular capacity, strength, agility, flexibility), heart rate, and blood pressure (Palacios et al. 2015). Currently, the standard practice for monitoring effect of exercise is by analyzing plasma or serum biochemistry. However, it may be more informative to study cells such as adult stem cells (ASCs) to observe the effect at a cellular level.

Why Adult Stem Cells

Stem cells are nonspecialized cells which can differentiate into specialized organ or tissue specific cells. Predominantly, stem cells are divided embryonic and somatic or adult stem cells. The roles of stem cells on embryonic development have remained the main focus in regenerative medicine for a long time compared to ASCs. ASCs can be found in a variety of tissues including bone marrow, peripheral blood, blood vessels, skeletal muscle, adipose, and brain by forming niche for specific and multitasking work. For Human Endothelial Progenitor Cells (EPCs) and MSCs, bone marrow is the main reservoir. Peripheral blood contains approximately 1% of MNCs as EPCs. MSCs can be obtained from bone marrow, and adipose tissue has also been well established for MSC production (Hass et al. 2011). Predominantly, ASCs are responsible for daily tissue or cell maintenance, remodeling and regeneration of multiple tissues, and especially to replenish cell death from apoptosis (Yoder 2012). Often, adult stem cells (ASCs) respond to tissue-specific signals by migrating to a closer proximity of the injury site. Exercise can influence these adult stem cells function and fate by altering extracellular matrix composition, and inflammation

(Van Craenenbroeck and Conraads 2010). In addition, exercise also promotes migratory capacity of stem cells.

Effect of Exercise on Endothelial Progenitor Cells (EPCs)

Effects on EPC

EPCs are circulating cells, abundantly available in peripheral blood, bone marrow, and umbilical cord. Most commonly, EPCs are defined by cell surface markers such as CD34+ or CD34+/KDR+ or CD34+/KDR+/CD133+. EPCs play an important role in angiogenesis and neovascularization, predominantly, by incorporation into the endothelium or by its paracrine properties that favor de novo vessel formation (Boppart et al. 2015). CVD and diabetes often lead to vascular damage, and EPCs play a vital role in repair and regeneration of blood vessels. The benefit of physical activity can be enhanced by healthy nutrition, and both can synergistically help to prevent or reverse the outcome of poor CVD outcomes of diabetes (Francois et al. 2018).

Metabolic disorders like diabetes dramatically decrease EPC number and impair its function. On the other hand, it is well established that the increase of EPC number and function is beneficial for both diabetes and CVD. Physical activity increases the production and increases circulating numbers of EPCs. Studies have showed that EPC production is partially dependent on nitric oxide (NO) production that may be secondary to its anti-apoptotic effect (Laufs et al. 2004; Steiner et al. 2005; Jenkins et al. 2009). Similarly, it is also reported that exercise helps to reduce PI3-kinase-mediated apoptosis, which depends on nitric oxide. PGE1-mediated upregulation of EPC is also linked to the improvement of EPC function and improved angiogenesis (Gensch et al. 2007). Another study showing improvement of EPC number may be related and preceded by an increase in plasma VEGF. They showed in patients with coronary artery disease (CAD), exercise induces a short-term myocardial ischemia which increases EPC numbers, depending on VEGF (Adams et al. 2004a). In addition to this study, patients with chronic heart failure show increased EPC counts by increasing not only plasma VEGF but also SDF-1 levels post exercise (Sarto et al. 2007). Rise of CD34+/VEGFR2+ cell number may be influenced by alterations in oxidative stress (Witkowski et al. 2010).

Age Is Another Major Factor Behind Reduction of EPC Numbers

The CD34+/KDR+EPC numbers are two times higher in younger patient populations compared to older groups at the resting state. Studies showed that exercise increased the number of EPCs in middle age and older persons (Hoetzer et al. 2007). Interestingly, another study showed exercise increased the number of EPCs independent of age group (Thijssen et al. 2006).

Exercise not only increases the number of EPCs but also improves the function of EPCs. Sen et al. (2015) showed a 6-week exercise program improves $CD34^{+}$ cell function by increasing the migration of EPCs to the chemotactic factor SDF-1α which leads to enhanced vasculoneogenesis (Sen et al. 2015). It is also reported that exercise substantially influenced SDF-1α levels over time (Van Craenenbroeck et al. 2011). In addition, studies have shown atherosclerotic plaque decline in response to exercise (Ajijola et al. 2009). It has been reported that supervised exercise training (SET) boosts the circulating EPC counts and reduces asymmetric dimethylarginine (ADMA) levels that leads to increased angiogenesis and improved endothelial function and decrease atherosclerosis. Therefore, cell mobilization induced by exercise and reduction of ADMA may serve as a physiologic mechanism repair for atherosclerosis (Schlager et al. 2011; Van Craenenbroeck et al. 2010; Rehman et al. 2004).

In patients with hypertension, exercise reduces impairment of EPC, which promotes neovascularization to heal injury as a result of chronic hypertension (Fernandes et al. 2012). In an interesting review Wahl et al. summarized the process of EPC function. They discussed exercise promoting mechanical stress to the tissue and vasculature. This mechanical force directly or indirectly regulates EPCs fate. Collectively, exercise promotes the release of growth factors and other molecules like IL6 and NO which facilitate EPC differentiation and production from bone marrow and also helps to promote migration and homing in the hypoxic tissue to improve angiogenesis and vasculogenesis (Wahl et al. 2008).

Expectedly, the benefit of exercise is time-dependent. Duration of exercise directly manipulates circulating EPCs numbers (Adams et al. 2004b). It has been reported that intensive and moderate exercise activity for 30 minutes increases circulating EPC number but this outcome is not seen when the time of exercise is reduced to 10 minutes (Laufs et al. 2005) and that a maximal bout of exercise stimulates a significant shift in CD34+ cells toward CD34+/KDR+ cells.

Effect of Novel Diabetes Medications on Endothelial Progenitor Cells (EPCs)

As mentioned above, EPCs can act as a cellular biomarker that is more reliable than serum-based markers for estimating and following endothelial dysfunction in early type 2 diabetes patients. Thus, investigating EPCs could help develop a cardiovascular disease (CVD) risk estimation (Adams et al. 2004b; Laufs et al. 2005; Dore et al. 2018).

Dipeptidyl peptidase-4 (DPP-4) inhibitors, a popular class of antidiabetic medications, have been shown to achieve improved glycemic control by lowering HbA1C, without causing hypoglycemia, and are weight neutral (American Diabetes Association 2013; The Diabetes Prevention Program Research Group 2002; Center for disease control and prevention 2015). Because DPP-4 degrades particular incretins, such as SDF-1α, its inhibition is also linked with a potential mechanism to prevent vascular diseases. However, there are limited data demonstrating the

potential cardiovascular effects of these medications. Only a few studies using either sitagliptin or saxagliptin have shown an increase in endothelial progenitor cells, and thus potential cardiovascular benefits, with DPP-4 therapy.

Metformin has commonly been used as the first-line pharmacologic agent for treating diabetes and prediabetes as per the American Diabetes Association guidelines. Metformin improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption in the intestines and stomach, and increasing insulin-mediated glucose uptake (American Diabetes Association 2013; The Diabetes Prevention Program Research Group 2002; Center for disease control and prevention 2015). Metformin has shown cardioprotective effects by increasing endothelial progenitor cells and CFU-Hill's colonies in type 1 diabetes, and is known to also have cardioprotective properties in type 2 diabetes (Dore et al. 2018).

The upregulation of SDF-1α and vascular endothelial growth factor (VEGF), both chemotactic factors in serum, increases mobilization and recruitment of EPCs in the face of acute ischemic injury for repair and regeneration (Dore et al. 2018). It is postulated that DPP-4 inhibitors may increase EPCs mobilization from the bone marrow via their role in increased SDF-1α presence in serum. Since poor viability and impaired function of EPCs in early diabetes will ultimately affect the repair and regeneration of the endothelium, prompt intervention may help to reduce or reverse cardiovascular risk by improving EPCs survival and function above and beyond adequate glucose metabolism control.

We conducted a study (Dore et al. 2018) where we investigated whether the addition of *saxagliptin,* a DPP-IV inhibitor, to metformin affects the number, function, and gene expression of peripheral blood-derived CD34+ cells in addition to improving glycemic control in Type 2 diabetes patients. In this 12-week, double-blind, randomized placebo-controlled trial, 42 subjects already taking metformin 1–2 grams/day were randomized to placebo or saxagliptin 5 mg. Subjects aged 40–70 years with diabetes for <10 years, with no known cardiovascular disease, BMI 25–39.9, HbA1C 6–9% were included. We evaluated EPCs' number, function, surface markers, and gene expression. We also investigated arterial stiffness, blood biochemistries, resting energy expenditure, and body composition parameters. A mixed model regression to examine saxagliptin vs placebo, accounting for within-subject autocorrelation, was done with SAS ($p < 0.05$).

Although there was no significant increase in CD34+ cell number, CD31+ cells percentage increased. Saxagliptin increased migration (in response to SDF1α) with a trend of higher colony formation count. MNCs cytometry showed a higher percentage of CXCR4 double positivity for both CD34 and CD31 positive cells, indicating a functional improvement. Gene expression analysis showed an upregulation in CD34 + cells for antioxidant SOD1 ($p < 0.05$) and a downregulation in CD34- cells for IL-6 ($p < 0.01$). For arterial stiffness, both augmentation index and systolic blood pressure measures went down in saxagliptin subjects ($p < 0.05$). We concluded that Saxagliptin, a novel DPP4 inhibitor when administered orally, in combination with metformin, can help improve cellular and biochemical parameters of endothelial function in early diabetes before macrovascular complications appear. We also established that CD34+ cells can act as a valuable biomarker (Dore et al. 2018).

We conducted another study (Nandula et al. 2021) using a novel agent from a class of diabetes medications called *sodium glucose channel inhibitors (SGLT2i) such as Canagliflozin (CG) on CD34+ve progenitor cells*. We investigated whether the addition of canagliflozin (CG), an SGLT2 inhibitor to metformin and/or insulin, would affect the number, function, and gene expression of peripheral blood-derived CD34+ cells in addition to improving glycemic control in Type 2 diabetes patients.

In this pilot study 29 subjects taking metformin and/or Insulin were enrolled in a 16-week, double-blind, randomized placebo matched trial, with a low dose 100 mg CG as the intervention group compared to matched placebo. Type 2 diabetes subjects (30–70 years old), with hemoglobin A1c (HbA1c) of 7–10%, were enrolled. CD34 +ve cell number, migratory function, gene expression along with vascular parameters such as arterial stiffness, serum biochemistry pertaining to cardiometabolic health, resting energy expenditure, and body composition were measured. Data were collected at week 0, 8, and 16. A mixed model regression analysis was done, and p-values less than 0.05 were considered statistically significant. Our results showed that a significant expression of CXCR4 receptor (a cell surface receptor for a prominent chemotactic factor stromal derived factor-1) with a concomitant increase in migratory function of CD34+ve cells was observed in CG treated group as compared to the placebo group. Gene expression analysis of CD34+ve cells showed an increase in expression of antioxidants (superoxide dismutase 2 or SOD2, Catalase, and Glutathione Peroxidase, or GPX) and notable endothelial markers (PECAM1, VEGF-A, and NOS3). A significant reduction in glucose and HbA1c levels were observed along with improved systolic and diastolic blood pressure in the CG group. A significant increase in adiponectin ($p = 0.006$) was also noted in the treatment group. Urinary exosomal protein leak in urine, examining podocyte health (podocalyxin, Wilm's tumor, and nephrin), showed a reduction with CG. We concluded that even at a low dose Canagliflozin has a beneficial effect on CD34+ cell function, serum biochemistry, and urinary podocyte-specific exosomes in type 2 diabetes.

Effect of HIV Medication Regimens on Endothelial Progenitor Cells (EPCs)

With these promising results using CD34+ cells as a biomarker in type 2 diabetes, as described above, we went on to investigate the effect of different therapeutic regimens used in another chronic disease that causes systemic inflammation, metabolic dysfunction including lipodystrophies, and cardiovascular disease such as HIV (Elzarki et al. 2022) in subjects without diabetes.

Our goal was to determine the effects of an integrase inhibitor (INSTI) in comparison to non-INSTI based regimens such as non-nucleoside reverse transcriptase inhibitors (NNRTIs)-based regimens on cardiovascular disease (CVD) risk in HIV+ patients without an overt history of CVD or diabetes, with normal CD4:CD8 count. For CVD risk assessment, we primarily used hematopoietic CD34+ progenitor cells, as a biomarker. We enrolled 19 male subjects ages 32–61 years with BMI

21.0–36.0. This was a single time point, cross-sectional, observational study. Subjects were enrolled under two groups (either on INSTI-based regimen with 13 subjects or NNRTI (non-INSTI)-based regimens with 6 subjects) who were taking stable doses of HAART. The medication regimens were a combination of one NRTI (typically tenofovir-emtricitabine) plus one INSTI or NNRTI. Our outcome measures were focused on cardiovascular and endothelial cell function and systemic inflammation. Our primary outcome measures were peripheral blood-derived hematopoietic progenitor cell number (CD34 and CD133 positive), CD34+ cell function, and gene expression studies. Our secondary outcomes were arterial stiffness measures and serum-based markers of inflammation. Our results indicated a significant increase in percentage number of progenitor cells, and CD133+ cells ($p = 0.004$) along with an increase of double progenitor mark positive CD133+/CD34+ progenitor cell population was observed in INSTI group as compared to NNRTI group, by flow cytometry. mRNA gene expression for antioxidant gene catalase along with a trend toward a decrease in gene expression of inflammatory marker IL6 ($p = 0.06$) was observed in CD34+ from INSTI group vs NNRTI group. The plasma IL-6 and CRP levels did not change significantly between the groups. Neutrophil-lymphocyte ratio (NLR), an important marker of inflammation, was noted to be lower in the INSTI group. A mean fasting glucose level was also lower in the INSTI group compared to NNRTI group ($p = 0.03$). Interestingly, Urine- Microalbumin levels were higher in the INSTI group compared to NNRTI group ($p = 0.08$), while eGFR levels were significantly lower in the INSTI group ($p = 0.002$). The arterial stiffness measures did not show statistically significant differences between the two groups. We concluded that the INSTI regimen may provide a better CVD risk profile compared to NNRTI-based HAART regimen; however, the increased albuminuria along with lower eGFR noted in INSTI group is of concern. Similar to other studies (Dore et al. 2018; Nandula et al. 2021) this study demonstrated that noncellular results (both plasma values and microalbuminuria) did not show a statistically significant difference, during the short period of study/intervention however our cellular results, based on CD34+ cells actually did show statistically significant differences in number and gene expressions assays. Our results once again demonstrated that cellular results may be of more value even if the cohort size is small, compared to noncellular results, though the latter is more commonly used clinically (Elzarki et al. 2022).

Effects on Exercise on MSCs

Mesenchymal stromal cells (MSCs) are multipotent cells that can differentiate into osteoblasts, adipocytes, and chondrocytes. Sources of obtaining MSC vary from umbilical cord blood, bone marrow, adipose tissue, pancreatic islet, fetal liver to the lung (Zuk et al. 2001; Agricultural and biological sciences “Genetic engineering - an insight into the strategies and applications” 2016). Often, MSCs are defined by specific markers such as CD44, VD73, CD90, CD105 but not CD31, CD34, CD45 (Dominici et al. 2006). Studies have showed that exercise may facilitate MSC

migration by increasing IL6 and recruiting stem cells at the site of injury (Schmidt et al. 2009). It was reported that the MSC secretome is responsible for hematopoietic stem and progenitor cells (HSPC) mobilization and proliferation and exercise induce homing of HSPCs to extramedullary sites (Emmons et al. 2016).

Often, the effect of MSC transplantation increased with exercise. Shin et al. showed treadmill exercise increased therapeutic MSC transplantation in traumatic brain injury (TBI) in a rat model (Shin et al. 2016). It has also been reported that exercise increased the efficiency of MSC transplantation in cerebral ischemic rats by reducing apoptosis (Zhang et al. 2015). Another study showed stromal vascular fraction, a well-known mixed population enriched with MSCs, and exercise together help to improve pain in patients with knee osteoarthritis (Gibbs et al. 2015). Another group of scientists reported that exercise facilitates MSC transplantation in idiopathic osteonecrosis (ION) of the femoral head by increasing vascularization in bone graft (Aoyama et al. 2015).

Exercise plays a vital role in differentiation of multipotent MSCs. It is reported that exercise promotes bone differentiation in MSCs. Li et al. indicate mechanical force increased osteogenic differentiation by increasing RUX2 and OXS, and at the same time, it is responsible for the reduction of PPARγ-2 and C/EBPα which indicates reduced adipogenesis (Li et al. 2015). The effect of exercise on osteogenesis was observed for bone marrow-derived MSCs (Liu et al. 2017). Another study that we conducted showed exercise promotes osteogenic differentiation in the veteran population post-exercise. Interestingly, bone differentiation markers such as RUNX, ALPL, and osteocalcin upregulated significantly which indicates osteogenic differentiation (Kundu et al. 2019). Cook et al. discussed how signaling pathways manipulate MSC differentiation. Both BMP and WNT signaling pathways play an important role in MSC differentiation. WNT signaling promotes osteogenic differentiation by upregulating RUNX and inhibiting PPARγ. On the other hand, BMPs activate osteogenic differentiation by activating RUNX (Cook and Genever 2013).

Maredziak et al. showed exercise is increasing bone marrow-derived MSC number. This group also reported elevated alkaline phosphatase, osteopontin, and osteocalcin which directly indicate increased musculoskeletal function (Marędziak et al. 2015) in mice. It is reported that exercise promotes activation and differentiation of skeletal muscle cells by promoting growth factors such as IGF-1, MGF, FGF, HGF, IL6, and signaling molecule NO (Wahl et al. 2008). It is also reported that exercise may improve cartilage repair followed MSC transplantation (Yamaguchi et al. 2016).

Similar to exercise, weight-reducing diabetes medications may also alter the MSC differentiation pathway. However, placebo-matched studies to elucidate such effects are warranted. Therefore, the MSC differentiation pathway away from adipogenesis and toward osteogenesis may act as a useful biomarker to monitor the effect of exercise and diabetes medications in subjects with diabetes. This may usher in assessing individual responses to exercise and diabetes medications.

Other biomarkers that may be important to highlight *include extracellular vesicles or exosomes* in serum and urine to monitor complications of diabetes. In

our study, we quantified urine exosome-based podocyte proteins in order to monitor podocyte inflammation (in the context of diabetic nephropathy) secondary to diabetes medications (Nandula et al. 2021; Awal et al. 2020).

Podocytes play a critical role in the formation of the glomerular filtration barrier and damage to these specialized terminally differentiated epithelial cells leads to proteinuria and progressive chronic kidney disease. Research suggests urinary exosomes are a more sensitive biomarker of early injury to podocytes being detected in the urine before traditional measures including albuminuria and total proteinuria (Burger et al. 2014; Kwon et al. 2017; Gilani et al. 2017). Recent studies show urinary exosomes of podocyte-specific proteins are increased in early diabetic kidney disease (Burger et al. 2014), preeclampsia (Kwon et al. 2017), and renovascular disease (Gilani et al. 2017). Additional research is needed to determine sensitive biomarkers for early diabetic injury. Significantly, these biomarkers can potentially assist clinicians in identifying the patients who would most benefit from the multitude of newly approved therapeutic agents for treatment of diabetic kidney disease including SGLT2 inhibitors, GLP-1 agonists, and mineralocorticoid receptor antagonists.

Another important biomarker that appears to change in response to alterations in metabolism appears to be the *study of gut microbiome*. Gut microbiome in combination with artificial intelligence and machine learning could be the cutting-edge technology for prediction of disease progression such as prediabetes to diabetes or even prediction of positive response to a medication intervention, in the context of diabetes therapeutics (Hopson et al. 2020). Machine learning and novel biomarkers may also help in our fight toward the global COVID-19 pandemic, a disease which seems to have a significant association with metabolic dysregulation (Gogate et al. 2021; Gogate et al. 2020; Kaviani et al. 2022).

Conclusion

Adult progenitor/stem cells can prove to be an effective and efficient biomarker, not only to monitor exercise but also to monitor response to medications. They appear to be relatively unexplored in the realm of metabolic disorders.

Applications to Other Diseases or Conditions

Inflammatory diseases including obesity, diabetes, HIV, and cardiovascular disease have reached epidemic proportions worldwide and have a significant impact on morbidity and mortality. There is a critical need to identify biomarkers of these disease states to target therapies at an earlier stage prior to irreversible injury. Endothelial progenitor cells (EPCs, defined as CD34+) from hematopoietic lineage and adipose tissue-derived mesenchymal stem cells, or AD-MSCs, can serve as such a biomarker and can monitor response to therapeutic interventions including exercise and medications. Both EPCs and AD-MSCs are easy and safe to

obtain from subjects. Exercise can influence EPCs and ASC function by altering extracellular matrix composition, and inflammation. Exercise also promotes migratory capacity of adult progenitor and stem cells. Physical activity increases the production and function of EPCs. Duration of exercise directly manipulates the number of circulating EPCs. Exercise may also help to facilitate MSC migration by increasing IL6 and recruiting stem cells to the site of injury. Exercise plays a vital role in differentiation of multipotent MSCs. EPCs can act as a cellular biomarker for cardiovascular diseases that is more reliable than serum-based markers for estimating the degree of endothelial cell dysfunction in early type 2 diabetes patients. ASCs have the potential to revolutionize the management of patients with a variety of metabolic and obesity-related disorders and also pro-inflammatory diseases. Further investigation of clinical entities using EPCs and ASCs are urgently needed.

Mini-dictionary of Terms

Endothelial Progenitor Cells Circulating cells available in peripheral blood, bone marrow, and umbilical cord and are defined by cell surface markers such as CD34+ or CD34+/KDR+ or CD34+/KDR+/CD133+. They play an important role in angiogenesis and neovascularization.

Exosomes An extracellular vesicle that contains constituents (protein, DNA, and RNA) of the cells that produce them.

Gut Microbiome Microorganisms that live in the digestive system and have an impact on health.

Mesenchymal stromal cells Multipotent cells that can differentiate into osteoblasts, adipocytes, and chondrocytes and can be differentiated from bone, fat, and cartilage.

Stem Cells Nonspecialized cells which can differentiate into specialized organ or tissue-specific cells.

Key Facts

- Stem cells are divided into embryonic and somatic or adult stem cells.
- EPCs and MSCs are responsible for daily tissue or cell maintenance and replenishment of cell death from apoptosis.
- Both EPCs and MSCs (particularly AD-MSCs) can serve as key biomarkers of metabolic disorders, inflammatory diseases including the response to therapeutic interventions in these disorders such as exercise, dietary modifications, and medications.
- Exercise can influence both EPC and AD-MSC function by altering extracellular matrix composition, inflammation, and promoting the migratory capacity of stem cells.

- Other biomarkers being explored include plasma and urinary exosomes and also the gut microbiome.

Summary Points

- Obesity, prediabetes, diabetes, HIV, and cardiovascular diseases are worldwide epidemics that have a profound impact on morbidity and mortality.
- It is essential to monitor the effects of exercise and various therapeutic agents for the treatment of these diseases.
- Adult stem cells play a major role in remodeling of the body and tissue regeneration and may have an important role as a biomarker.
- EPCs and MSCs can serve as a biomarker of disease activity including in diabetes, obesity, HIV, and cardiovascular disease.
- These adult stem cells (ASCs) are easily obtained from peripheral blood or adipose tissue depots, and are precursors to endothelium and mesenchymal tissue.
- Other potential biomarkers to explore in this area include plasma/urine exosomes and the gut microbiome.
- Additional biomarkers for diagnosis and prognosis in chronic pro-inflammatory diseases are urgently needed to aid in the management of these increasingly prevalent cardiometabolic diseases such prediabetes, diabetes, and obesity.

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The Search for Immunological Biomarkers in Type 1 Diabetes Mellitus (T1DM) and Multiple Sclerosis (MS): Th40 Cells Provide a Common Autoimmune Link

27

David H. Wagner

Contents

Mini Dictionary of Terms 590
Key Facts 590
Summary Points 591
References 591

Abstract

Biomarkers to predict autoimmune disease onset or predict progression are sorely lacking. A specific T cell subset present in peripheral blood of human subjects and mouse models called Th40, $CD4^+$ helper T cells that express the CD40 receptor, are emerging as diagnostic in type 1 diabetes mellitus (T1DM) and multiple sclerosis (MS). Th40 cell number expansions predate symptoms in both T1DM and MS in human subjects. Th40 cells prove pathogenicity by transferring T1DM in the diabetes preclinical mouse model and transferring severe symptoms in the MS mouse model. Th40 cells undergo a process called TCR revision, altering TCR expression in the periphery, constituting a mechanism that increases immune diversity to invaders like viruses, but also may increase the existence of self-antigen reactive T cells. Th40 cells are indicative of systemic inflammation and in combination with other markers, HLA and chemokine receptor expressions, can be diagnostic for specific autoimmune diseases.

Keywords

Biomarker · Th40 cell · Type 1 diabetes mellitus · Multiple sclerosis · T cell receptor revision

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Abbreviations

AAb	Autoantibody
APC	Antigen presenting cell
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DKA	Diabetic ketoacidosis
GAD	Glutamic acid decarboxylase
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
IAP	Islet-associated peptide
IFNγ	Interferon gamma
IL	Interleukin
INS	Insulin
LADA	Latent autoimmune diabetes in adults
MHC	Major histocompatibility complex
MS	Multiple sclerosis
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCR	T cell receptor
Th	T helper
TNFα	Tumor necrosis factor alpha
TREC	T cell receptor excision circle

A central tenet of autoimmune disease is that cells of the immune system become activated to attack self-tissue resulting in often debilitating tissue damage. Normally immune cells identify and eliminate pathogens including viruses, bacteria, and fungus. Type 1 diabetes mellitus (T1DM) arises because immune cells attack the beta cells within pancreatic islets leading to loss of insulin production. Multiple sclerosis (MS) arises because immune cells attack the central nervous system leading to loss of myelin, the neuronal protective sheath. Rheumatoid arthritis (RA) arises when immune cells target and damage synovial tissue in joints. Other diseases including Hashimoto's thyroiditis, ulcerative colitis and Crohn's disease, lupus, etc. similarly involve tissue-related attacks. While each autoimmune disease presents with a primary pathology all have overlapping secondary pathologies. For example, T1DM, MS, RA, and lupus, all result in cardiovascular disease complications including high risk for atherosclerosis, coronary artery disease, and peripheral artery disease (Schofield et al. 2019; Haubitz 2007; Magyari and Sorensen 2020; Logstrup et al. 2020; Semb et al. 2021). These examples demonstrate how pernicious autoimmunity becomes, i.e., pathologies extending beyond the characteristic, diagnostic pathology. Secondary pathologies involve the same immune cell types that initiated the original pathology.

A major shortfall throughout autoimmune diseases is reliable biomarkers that predict disease onset, predict disease progression, or possibly indicate disease

remission. Biomarkers that indicate any of these disease transitions would improve treatment options or indicate necessary treatment option changes that would improve overall outcomes. The general approach has been to focus on specific tissue-related biomarkers associated with individual autoimmune diseases. Arguably, a more comprehensive biomarker approach, e.g., examination of immune cell potential biomarkers in multiple autoimmune diseases, may yield more useful information. An immune cell type, $CD4^+$ helper T cells that express the CD40 receptor and hence referred to as Th40 cells, has arisen as a common feature in multiple autoimmune diseases (Vaitaitis et al. 2010, 2013, 2014, 2017a, b, 2019a, b; Wagner 2017; Amit et al. 2016; Liu et al. 2015; Waid et al. 2008, 2014; Deng et al. 2014; Torres et al. 2014; Vaitaitis and Wagner 2008, 2010, 2013; Van Eenennaam et al. 2002). This review explores how Th40 cells develop, relate to autoimmunity specifically focusing on T1DM and MS, and drive auto-inflammation. A more specific approach for associating biomarkers with specific autoimmune diseases is proposed.

The search for unique biomarkers – type 1 diabetes mellitus (T1DM): Because of the unique pathologies associated with individual autoimmune diseases the search for biomarkers has focused on the specific pathology. T1DM biomarker searches have focused on pancreatic islet markers; ideally that would be detected in blood, allowing easy access. The classic initial diagnostic is consistently elevated serum glucose that eventually spills into urine. T1DM symptoms include increased fatigue, polydipsia, polyuria, and occasional quotidian fever; all are indirectly related to persistent hyperglycemia. However, glucose elevations occur independently of diabetes. During high stress, release of catecholamines and stress hormones stimulate glucose production and interfere with tissue disposal of glucose (Halter et al. 1984). Consistently elevated blood glucose and a failed oral glucose tolerance test are necessary indicators of diabetes. Like T1DM, type 2 diabetes mellitus (T2DM) involves similar symptoms and hyperglycemia. The clinical difference is that T1DM arises from autoimmune damage to islet beta cell insulin production and T2DM involves insulin resistance. Inflammatory pathways are associated with T2DM, but it is not considered autoimmune. One major issue is that type 1 and type 2 are misdiagnosed surprisingly too often. Even with advances in current diagnostic capabilities, a retrospective study of 3030 diabetic patients found misdiagnosis occurred in 25% of participants (Munoz et al. 2019). Other individual studies have found misdiagnosis as high as 35% (report from the American Diabetes Association). The consequences of misdiagnosis can be severe with diabetic ketoacidosis (DKA) more likely to arise when diabetes is misdiagnosed; DKA poses severe consequences to patients including significantly increased risk of death (Bao et al. 2019). In fact, DKA is the leading cause of death in pediatric T1 DM (Munoz et al. 2019; Peng et al. 2021).

Genetic contributors have been identified in T1DM. The most highly associated genes are the human leukocyte antigen (HLA) loci (Pugliese et al. 2016; Barrett et al. 2009; Valdes et al. 2005). T1DM associates with *HLA-DRB1*03,* (DR3), *DRB1*04,* (DR4) *DQA1*05:01-DQB1*02:01* (DQ2), and *DQA1*03-DQB1*03:02* (DQ8). Genome-wide association studies (GWAS) also indicate CLTA-4, PTPN22, and IL2RA1 as contributory (Barrett et al. 2009). Adding genome-wide SNP typing

analysis an additional 19 loci including IL-2, IL-7R, TNFAIP3, CD226, IL-18R, and CCR5 were identified (Barrett et al. 2009). Except for the insulin locus, all other loci associate with effector immune cells that are found in other autoimmune diseases, making specificity difficult. The discovery of the involvement of the insulin locus in GWAS led to a series of four different clinical trials attempting to use insulin or insulin-derived peptides for tolerance therapy; all those trials failed (Bleich and Wagner 2018). Further studies developed small peptides derived from suspected contributor proteins such as GAD65 (Mangada et al. 2009) to attempt tolerance induction. These tolerance induction approaches were successful in the NOD mouse model; yet to date, none have translated to the human clinic.

One biomarker proposal came after the observation that during T1DM autoantibodies (AAb) were detected in peripheral blood. Antibodies that recognize insulin (INS), GAD65, IA-2, or ZnT8 (zinc transporter) have been associated with diabetes development and are presumed to be unique to diabetes. Some diabetic subjects and some first-degree relatives of T1DM subjects demonstrated one or more of those AAbs. With the greater number of AAbs detected, it was presumed that the risk of developing T1DM increased. Retrospective study showed however that detection of multiple AAbs was highly diagnostic in children 5 years of age or younger, but diagnostic significance rigor in older children failed (Battaglia et al. 2015; Lampasona and Liberati 2016; Michels et al. 2015). This also was true for adult onset T1DM. The average age of diabetes onset in 2011 for children was reported to be 9.25 down from 10.5 years of age (Sella et al. 2011), thus true juvenile diabetes onset is getting younger. Adult T1DM or latent autoimmune diabetes in adults (LADA) is increasing very rapidly and average age of onset is 35 years (Weber et al. 2011). While many hopes were pinned on AAbs as high-quality diagnostic tools, the results have proven disappointing.

The search for unique biomarkers – multiple sclerosis: Multiple sclerosis (MS) is a neurodegenerative disease that is classically autoimmune. T cells, B cells, and macrophages infiltrate the central nervous system (CNS), establish inflammation, and create lesions. The etiology remains unknown although like T1DM, genetic predisposition with environmental contributors and immune cell activation and dysfunction are involved (Vaitaitis and Wagner 2008). Clinically, MS is characterized by discrete episodes of neurologic dysfunction. The severity and the type of attack varies between patients. Diagnosis requires a series of clinical observations and magnetic resonance imaging (MRI) scans that define lesions located mainly in the periventricular white matter, posterior fossa, spinal cord, and in subcortical locations of the brain (Logstrup et al. 2020). Diagnostic criteria for MS were established and require that lesions exhibit dissemination in time and/or space (Van Eenennaam et al. 2002; Halter et al. 1984). The variety of symptoms ranging from blurred vision, headaches, paraparesis, and severe paralysis reflect multifocal lesions and result from interruption of myelinated tracts in the CNS. The distinct disease courses include clinically isolated syndrome (CIS) defined by limited symptom occurrence (usually only one) but may include detection of one or more lesions by MRI (Van Eenennaam et al. 2002); approximately 50% of CIS progress to relapsing/remitting MS (RMS) (Van Eenennaam

et al. 2002). RMS accounts for approximately 80% of cases and is defined by recurrent neurologic symptoms with full or partial recovery and lack of disease progression between relapses (Van Eenennaam et al. 2002). Almost 50% of diagnosed RMS subjects will progress to secondary progressive MS (SPMS) by 15 years and approximately 90% convert to SPMS after 25 years (Van Eenennaam et al. 2002). SPMS is a neurodegenerative, continuous progression, e.g., lesion increases in time and space, and may involve occasional remissions or plateaus but always relapses afterward (Logstrup et al. 2020). Primary progressive MS (PPMS) is rarer and more neurodegenerative at onset with occasional plateaus but no sustained remission (Van Eenennaam et al. 2002).

Like T1DM, the best described biomarker thus far has been HLA haplotype. MS is associated with *HLA-DRB1*1501* (DR15 previously DQ2) and *DRB5*01-DQB1*0602* (DQ6) predominantly, it also is associated with DR4 and DQ2 (Waid et al. 2014). There are reports that T1D and MS have mutually exclusive HLA haplotypes, with T1D carrying an MS protective haplotype (Pugliese et al. 2016). However, recent findings of T1D and MS co-occurrence (Anaya et al. 2006; Marrosu et al. 2004; Tettey et al. 2015) that are continually expanding in number must be accounted for. Unlike T1DM, AAbs have not been described in MS. During MS, oligoclonal bands in the cerebrospinal fluid (CSF) were detected (Link and Huang 2006). However, the presence of oligoclonal bands in CSF occurs in patients with chronic or post-acute inflammatory diseases, including MS, of the central nervous system. Thus, oligoclonal bands are not unique to MS. The oligoclonal bands are IgG, the same immunoglobulin class as AAbs in T1DM, and for many years attempts to discover antigen specificity relative to CNS was attempted with no success (Giovannoni 2014; Hartung et al. 2019). A recent report found that the oligoclonal bands reacted to a range of individual peptides with epitopes sharing sequence homologies with proteins of viral origin, and proteins involved in cell stress, apoptosis, and inflammatory processes (Giovannoni 2014; Hartung et al. 2019). In other words, the oligoclonal bands IgGs react with proteins associated with tissue damage. The disappointment is that the IgGs are not CNS-specific antigens, the way AAbs in T1 DM are specific to known islet antigens. Like in MS, AAbs in T1DM appear to relate to tissue damage, not disease progression. Other molecules have been described in the CSF of MS patients including neurofilament, myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), tau protein, neuronal cell adhesion molecule (NCAM), and the growth-associated protein (GAP-43). However, the value of measuring these constituents for both diagnostic and prognostic purposes or for following response to therapy has not been determined (Magliozzi and Cross 2020).

Th40 cells – an autoimmune link: Historically, the CD40 receptor typically was associated with antigen presenting cells. It was first described on B cells, where it induces cell proliferation, and antibody class switching (Hollenbaugh et al. 1992). B cells also are an antigen presenting cell (APC) subclass and CD40 expression was later detected on macrophages and dendritic cells (DC), the other cells constituting APCs. In macrophages, CD40 engagement results in inflammatory cytokine production, nitric oxide production, and oxidative stress induction (Rizvi et al. 2008).

One of the identified ligands for CD40 is CD154 which was initially shown to be expressed on activated T cells. The original description of CD154 first called gp39 was T cell/B cell activation molecule (TBAM) (Foy et al. 1996; Foy et al. 1995). Because CD40 was first described on B cells, and its primary ligand was described on T cells, the inference was that their respective expression was cell type restricted, which turned out to be untrue. CD40 expression has been described in detail on T cells (Vaitaitis et al. 2010, 2013, 2014, 2017a, b, 2019a, b; Wagner 2017; Amit et al. 2016; Liu et al. 2015; Waid et al. 2008, 2014; Deng et al. 2014; Torres et al. 2014; Vaitaitis and Wagner 2008, 2010, 2013), neuronal cells, endothelium, and adipocytes, and CD154 is expressed on APC and platelets that are the primary source of CD154 (El Fakhry et al. 2012; Yashiro et al. 2009; Hassan et al. 2009; Alturaihi et al. 2015). A primary mechanism of action of CD40 on T cells is to induce inflammatory cytokines (Vaitaitis et al. 2014, 2017a, b, 2019b; Waid et al. 2014), induce RAG1 and RAG2, the gene recombination molecules responsible for rearranging T cell receptors (Vaitaitis et al. 2017b; Vaitaitis and Wagner 2013; Wagner 2016), and prevent Fas-mediated apoptosis in T cells isolated from autoimmune conditions (Vaitaitis and Wagner 2008, 2013; Waid et al. 2008).

Effector T cells carrying a specific T cell receptor (TCR) interact with specific antigens presented by antigen presenting cells (Fig. 1). The classic antigen presentation approach is that a resident APC takes up antigens and then processes and presents them through the major histocompatibility complex (MHC) class I or class II molecules (Fig. 1). The human immune equivalents are human leukocyte antigen (HLA) A, B, C (class I), and D (R, S, Q, U, class II). T cells sample the APC + antigen (Ag) complex and when the T cell carrying the appropriate TCR recognizes that APC/Ag, activation cascade is set in place. Step 1 of immune activation is antigen restricted and TCR expression dependent (Bretscher 1999). The activated effector T cells produce cytokines including IFNγ that is released into the milieu. As shown in Fig. 1, naïve T cells expressing the IFNγ receptor interact to induce CD40 (Kooy et al. 1999). CD40 expressing CD4 cells also develop in the thymus (Wagner et al. 1999, 2002; Carter et al. 2012; Wagner 2007) and survive positive and negative selection. In autoimmune-prone mice, thymic Th40 cells significantly ($p < 0.002$) are increased compared to non-autoimmune controls (Vaitaitis and Wagner 2013; Carter et al. 2012). Th40 cells express a broad repertoire of TCR molecules although clonal expansions within Th40 population occur in autoimmune diseases (Vaitaitis and Wagner 2013; Carter et al. 2012).

The TCR is composed of a discrete alpha and a discrete beta molecule that are generated and expressed during thymic development (Rubin and Kretz-Rommel 2001). The processes of positive and negative selection are meant to assure that only T cells carrying appropriately useful TCR molecules exit the thymus and populate the periphery (Romagnani 2006). This process fails occasionally, and self-antigen reactive T cells escape the thymus (Kishimoto and Sprent 2001). An additionally important and overlooked process that occurs in Th40 cells is TCR revision (Wagner 2016; Wagner and D.H. 2007; Kuklina 2006; Cooper et al. 2003). This is the process through which fully mature Th40 cells alter TCR expression in the periphery after exiting the thymus. Immunological dogma long has incorrectly

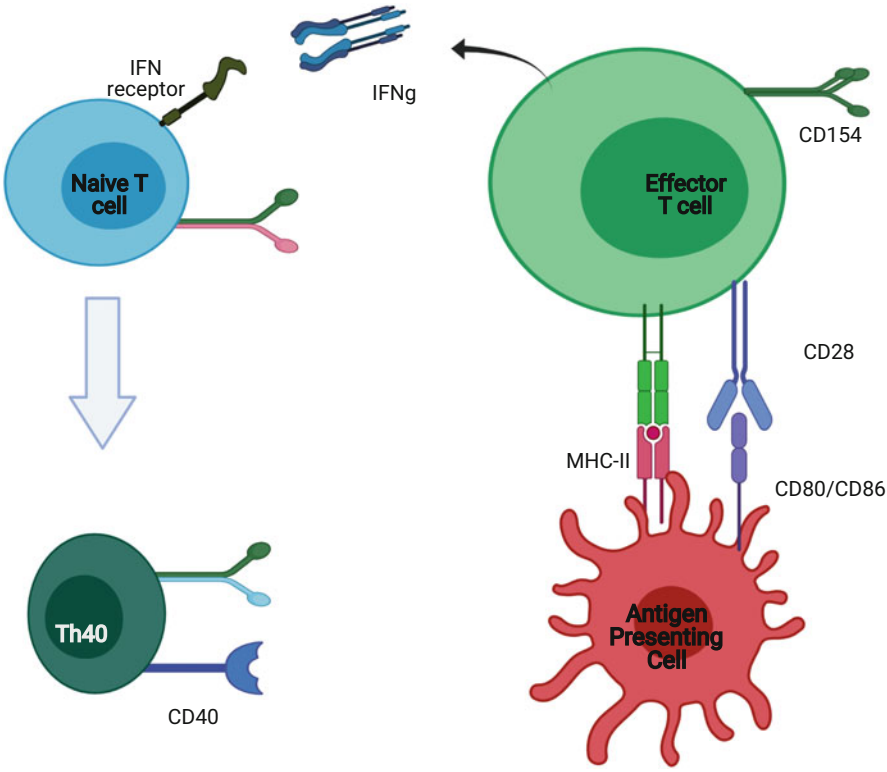

Fig. 1 ***Cartoon describing generation of Th40 cells***. Effector T cells interact with antigen presenting cells to become activated. Once activated the effector T cell releases IFNγ. Naïve T cells interact with IFNγ to induce CD40 expression, creating Th40 cells. (Figures created by Biorender)

held that once a TCR is expressed in the thymus, it cannot be altered. This dogma is not only illogical but disrupts basic tenets of conservation of biological energy.

A portion of $CD4^+$ T cells under inflammatory conditions become induced to express the CD40 receptor (Wagner 2017; Vaitaitis et al. 2014, 2017b) (Fig. 1). Another mechanism of action for CD40 on T cells is induction of the RAG1/RAG2 recombination machinery responsible for altering TCR expression (Vaitaitis et al. 2017b, 2003; Vaitaitis and Wagner 2013) (Fig. 2). When CD40 is engaged by CD154 (Fig. 2), the RAG proteins are induced (Vaitaitis and Wagner 2013; Vaitaitis et al. 2003; Waid et al. 2004). During thymic development, loci within the TCR genes, beta first then alpha, are bound by the RAG1/RAG2 heterodimer at specific sequences (Yannoutsos et al. 2001). A RAG heterodimer complex binds to a site and a second complex binds to a site further downstream. The two RAG heterodimer complexes come together creating a DNA loop. In the thymus, a splicease component of RAG1/RAG2 cleaves the DNA creating a TCR excision circle (TREC) (Zou et al. 2007). This event happens relatively randomly in each developing thymocyte,

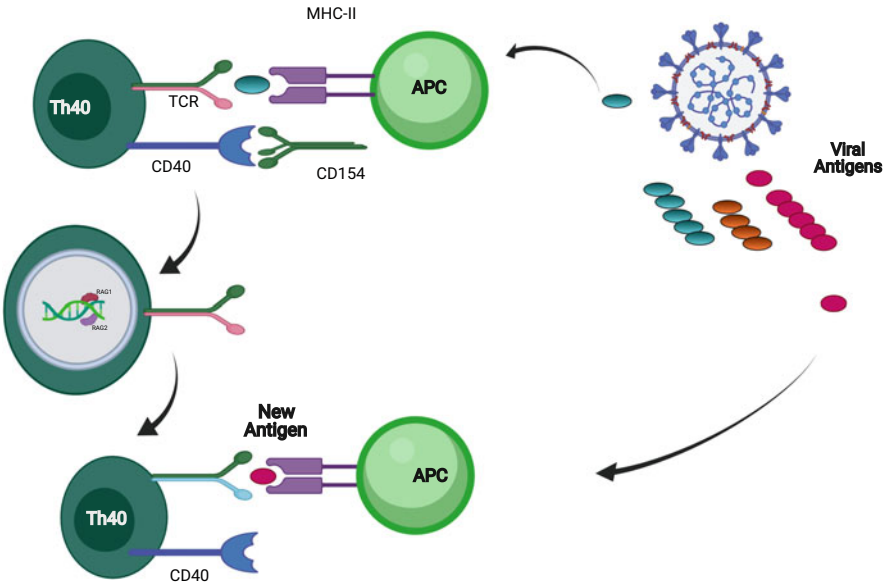

Fig. 2 ***T cell receptor revision***. Th40 cells express TCR alpha/beta molecules. When CD154 engages CD40 the RAG1/RAG2 proteins become induced. The heterodimer binds to DNA in the TCR locus and produces an alternate mRNA for TCR. The Th40 cell expresses a new TCR. Viral infections generate numerous antigens and when CD154 becomes available post-activation, conditions for TCR revision become strong. An altered TCR increases antigen recognition diversity.

thus any given thymocyte has a different TCR than its neighboring cells. This process creates diversity in the T cell repertoire. But because the entire gene is not spliced, additional alterations in the TCR genes can occur. In the thymus, second and third attempts at expressing novel TCR molecules is TCR editing (Santori et al. 2002). In peripheral Th40 cells, the RAG complex performs much the same process (Vaitaitis et al. 2003, 2017b; Vaitaitis and Wagner 2013; Wagner 2016). In peripheral T cell beta gene revision TRECs have been reported (Zou et al. 2007). In the alpha gene, TRECs were not reported (Vaitaitis et al. 2003, 2017b; Vaitaitis and Wagner 2013; Wagner 2016). The hypothesis was posed that in the periphery, the RAG complex binds TCR gene DNA in the same manner as in the thymus, with two of the complexes coming together to create looped DNA. It is hypothesized that rather than splicing the DNA, the RNA polymerase simply reads through the junction at the RAG complexes and the total DNA remains intact. This process preserves biological conservation, allowing TCR revision to occur more often and as needed, during a viral infection, for example.

Using a T cell clone with defined alpha (Va1)/beta (Vb4) molecules, after CD40 induced RAG1/RAG2 expression the clones expressed different TCR alpha and beta molecules (Vaitaitis et al. 2017b; Vaitaitis and Wagner 2013; Ali et al. 2003; McMahan and Fink 2000). TCR revision does not target recent thymic emigrants (Cooper et al. 2003), demonstrating that the process occurs in mature peripheral T

cells. TCR revision as a mechanism of action has both overall positive and negative consequences. During an infection when multiple foreign body antigens are generated, the ability to alter TCR expression is highly useful (Fig. 2). During a viral infection the natural immune response generates activated T cells that express CD154 (Jenkins et al. 2008). Antigen presenting cells and endothelial cells also express CD154 (Wagner 2017; Wagner et al. 2004).Thus a highly localized concentration of CD40 expressing T cells and of CD154 develops setting the stage for robust CD154–CD40 interactions including the ideal conditions for TCR revision (Fig. 2). This process is particularly beneficial given the mutability of viruses. There are reports that some individuals have protection against SARS-CoV-2 because of an exposure to a much less harmful corona virus variant or even to a previous virus of unknown origin (Balzan 2020). Protection to SARS-CoV-2 could have arisen from a previous viral exposure that caused TCR revision that consequentially resulted in a T cell capable of managing this corona virus.

The negative consequences of TCR revision include generation of autoantigen reactive T cells (Vaitaitis and Wagner 2013; Wagner 2007, 2016). Humans have evolved exceptional immunity to viruses given their myriad number and mutability making them such formidable foes. The price to pay for such strong viral immunity would be susceptibility to autoimmunity, under the current hypothesis. Because T cells remain susceptible to the rules of MHC interaction, the generation of TCR molecules in the periphery that do not conform to the rules of thymic positive selection will undergo death-by-neglect in the periphery. However, the constraints of negative selection, removal of self-antigen reactive developing T cells, may not apply in the periphery at least in all individuals. Peripheral negative selective pressures may occur in most individuals, but in those predisposed to autoimmunity, peripheral negative selective pressures would be lacking. Thus, a mechanism for development of autoreactive T cells in the periphery emerges. Clearly, the generation of a self-antigen reactive T cell in the periphery alone is not sufficient for autoimmune disease development. There have been multiple attempts over years to associate a particular virus with an autoimmune disease. For example, Epstein-Barr virus associated with MS (Tselis 2012), or coxsackie virus with T1D (Roep et al. 2002), etc. Rather than the proposed molecular mimicry hypothesis that does not survive fine statistical analysis, perhaps multiple viral exposures under autoimmune promoting genetic and environmental conditions are culprits. That is, multiple viral exposures leading to repeated TCR revisions thus generating a cadre of self-antigen reactive T cells provide an important component to autoimmune development under the right conditions. In this case, the right conditions are pathogenic, not evolutionarily advantageous. Again, this action alone would not be sufficient to establish autoimmunity, other components must be involved.

Th40 cells produce inflammatory cytokines through CD40, but through CD28 cells produce regulatory cytokines: Th40 cells behave like conventional T cells to produce cytokines when activated through the TCR/CD3 complex aided by co-stimulation (Vaitaitis et al. 2014, 2017a, b, 2019b; Waid et al. 2014). The classically defined conventional T cell co-stimulus molecule is CD28, although other T cell costimulatory molecules have been described (Wagner 2017). An

important mechanism of action for CD40 on Th40 cells is co-stimulation (Fig. 3). CD40 engagement on Th40 cells results in production and secretion of inflammatory cytokines and chemokines including IFN**γ**, TNFα, IL-1**β**, IL-6, IL-17a, IL-21, IL-22, MIP-1**α**, and MCP-1 (Vaitaitis et al. 2014, 2017a, b, 2019b; Waid et al. 2014). Interestingly, Th40 cells produce IFN**γ** and IL-17 concomitantly (Vaitaitis et al. 2013, 2017b; Waid et al. 2014). A study using the NOD mouse T1DM model showed that adoptive transfer of IFN**γ** producing T cells caused T1DM; IL-17 producing cells also caused T1DM but with delayed kinetics (Bending et al.

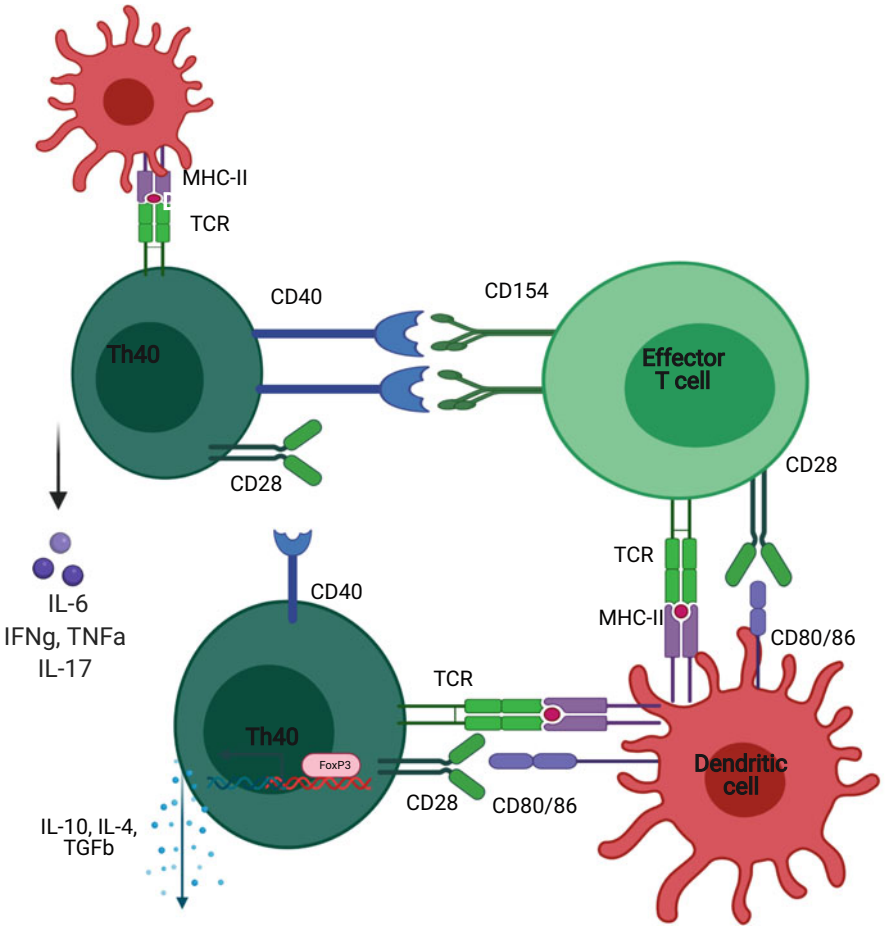

Fig. 3 ***CD40 drives inflammatory while CD28 drives regulatory cytokines***. An APC, dendritic cell, activates with an effector cell through TCR–MHC, signal 1, and CD28–CD80/CD86 interaction to express CD154. Th40 cells receive signal 1 signals through an APC. The rapid increase in CD154 provides a co-stimulus to Th40 cells to induce inflammatory cytokines. When the CD154 levels decrease as antigen is cleared, the Th40 cell can interact with a dendritic cell, receive signal 2 through CD28, and produce regulatory cytokines

2009). When those effector cells were isolated after diabetes onset, all IL-17 producing cells had converted to IFN**γ** producing cells (Bending et al. 2009). This finding suggests that in vivo Th17 cells are precursors to Th1 inflammatory cells. Th40 cells bridge the gap between Th17 and Th1 (Waid et al. 2014; Vaitaitis et al. 2013). When CD28 is engaged on Th40 cells regulatory cytokines including IL-10, IL-4, and TGF**β** are produced (Vaitaitis et al. 2014, 2019b; Waid et al. 2014) (Fig. 3). Co-engagement of CD40 and CD28 results in a mix of cytokines, some inflammatory and some regulatory, depending upon which signal is strongest, CD40 or CD28. This suggests that a quantitative difference in costimulatory molecule exposure dictates the direction of inflammatory versus regulatory conditions. Another interesting observation is that Th40 cells also can express FoxP3 (Vaitaitis et al. 2013; Waid et al. 2008), the transcription factor associated with regulatory T cells. Th40 cells exposed to TGF**p** became FoxP3$^+$ and CD40 engagement reduced FoxP3 expression (Vaitaitis et al. 2013). These observations suggest that rather than being exclusively regulated by classic Tregs, Th40 cells are capable of self-regulation. This opens alternate considerations for inflammatory versus regulatory conditions.

Alternate proposal for T cell inflammatory versus *regulatory cytokine polarization:* At exposure the virus capsid surface proteins dictate the cell types that will be infected through interaction with various proteins on the host cell surface. The virus enters the cell through receptor-mediated uptake. After initial infection, tissue-resident dendritic cells (DC) and macrophages (innate immune cells) uptake eventual viral antigens, and process and present those antigens on HLA-A, B, C, and D molecules. DCs and macrophages migrate to local lymph nodes to allow T cell sampling of antigens. The initial interaction in the lymph node will discover appropriate TCR bearing cells that become activated. Activated T cells proliferate and begin to produce IFN*γ* and other cytokines. IFN**γ** levels induce CD40 expression on naïve CD4$^+$ T cells creating Th40 cells (Fig. 1). Macrophages and DC at the site of infection become activated to secrete chemokines that will attract more T cells bearing the responsive chemokine receptor, from the regional lymph nodes. At the site of infection, Th40 cells carrying appropriately responsive TCRs interact with local APC and proliferate. Activated human T cells can express HLA molecules (Wilczynski 2006), therefore an activated T cell can interact with Th40 cells (Fig. 3). During this early stage of infection, CD154 and CD40 become abundant in the localized milieu. Th40 TCR interactions will be predominant from APC, but the large number of activated T cells can provide bystander levels of CD154. Thus, Th40 cells can interact directly with APC through TCR/CD3 and MHC but also CD40 on Th40 cells can interact with bystander CD154 from the activated T cells (Fig. 3). This process facilitates rapid production of moderate to high levels of inflammatory cytokines by Th40 cells.

An additional mechanism is that some of the Th40 cells undergo TCR revision as discussed earlier. This process increases T cell diversity at the site, improving response to the infection. Over time as antigen sources deplete, activated T cell numbers decrease, thus CD154 availability rapidly decreases and Th40 cells will now preferentially interact with CD80 (B7.1) and CD86 (B7.2) found on residual APC to reduce inflammatory cytokine production and increase regulatory cytokine

production (Fig. 3). Also, revision will stop. As the infection is controlled, Th40 cells recede in number and a portion convert to memory phenotype (Vaitaitis et al. 2019a; Waid et al. 2014). As stated above, a negative consequence of TCR revision is that some of the revised T cells can be self-antigen responsive. If this happens in a subject who is predisposed to autoimmunity, along with developing self-reactive T cells, additional autoimmune contributors facilitate the development of autoimmune disease. This hypothesis accounts for association of various viruses with autoimmune diseases as has been proposed multiple times. A major difference in this hypothesis versus previous suggestions is that any virus would create the conditions described above. Therefore, EBV could perform these actions in subjects who develop MS, and coxsackie B could do the same in eventual T1DM, but so could SARS-CoV-2, influenzas, etc. In fact, there are now descriptions of COVID-19 subjects developing autoimmune conditions (Taherifard et al. 2021; Zamani et al. 2021; Khamsi 2021; Nersesjan et al. 2021; Tariq et al. 2021; Dotan et al. 2021; Dalakas 2020; Grabbe et al. 2020). The scenario described above would contribute to autoimmune development, but multiple additional factors would also be required for autoimmune diseases to develop.

Th40 cells in T1DM: Th40 cells first were related to autoimmune disease using the NOD mouse model for T1DM (Vaitaitis et al. 2003, 2013, 2014, 2017a; Vaitaitis and Wagner 2008, 2010, 2012, 2013; Waid et al. 2004, 2007, 2008; Wagner et al. 2002; Carter et al. 2012). NOD mice spontaneously develop T1DM although with some sex bias; females generally have 80% incidence and males exhibit about 25% incidence, which is opposed to human T1DM that shows no sex bias. NOD mice diabetes development is like human, with progressive infiltration of pancreatic islets by immune cells leading to loss of insulin. In 3-week-old NOD mice, prior to insulitis, there is an expansion of Th40 cells localized to the pancreatic lymph nodes (Waid et al. 2004). As mice progress toward T1DM, levels of Th40 cells in the periphery expand in number (Waid et al. 2004). Well prior to diabetes onset, Th40 cells can be isolated directly from pancreatic islets. Pathogenicity was confirmed by adoptive transfer of Th40 cells from diabetic mice to NOD/SCID recipients (Vaitaitis et al. 2013, 2017a; Vaitaitis and Wagner 2008, 2010, 2013; Waid et al. 2004, 2008, Wagner et al. 2002; Carter et al. 2012). Also, Th40 cells isolated from prediabetic mice transfer diabetes. Th40 cells did not require activation and the majority achieved memory T cell status (Vaitaitis et al. 2010; Carter et al. 2012; Vaitaitis and Wagner 2012). Examination of human T1DM subjects showed that peripheral blood Th40 cell numbers were significantly expanded compared to non-autoimmune healthy controls, and the majority of Th40 cells were memory phenotype (Vaitaitis et al. 2019b; Waid et al. 2007; Siebert et al. 2008). The Th40 cell expansion was independent of HLA haplotype. That is, if a subject was diabetic, regardless of haplotype, Th40 cells were expanded. In HC that carried haplotypes associated with T1DM, Th40 cell numbers remained at control levels (Waid et al. 2007). Th40 cells from human diabetic subjects responded to islet antigens including insulin peptides, GAD65 peptide, and responded robustly to human islets (Waid et al. 2007). TrialNet, a study from the NIH and the American Diabetes Association, along with JDRF, is conducting a pre-T1DM natural history study. First-degree

relatives and subjects carrying at risk haplotypes are recruited. Th40 levels were measured in 65 pre-T1DM subjects (Vaitaitis et al. 2019b). The peripheral blood Th40 cell numbers represented a range, with some subjects having Th40 cell numbers equivalent to T1DM patient samples, and others at HC levels (Vaitaitis et al. 2019b). As with T1DM there was a wide range of HLA haplotypes represented in all pre-T1DM subjects. The older pre-T1DM subjects, potentially pre-LADA, within the DR4/DR4 or DQ8/DQ8 groups were more likely to have increased Th40 cell numbers (Vaitaitis et al. 2019b). This suggests combinational pre-LADA biomarker(s).

Th40 cells in multiple sclerosis: Like T1DM, MS develops over time and like T1DM, the strongest genetic link for MS is HLA haplotype. HLA-DRB1*1501, HLA-DQB1*0602, and HLA-DQA1*0102 alleles are strongly associated with MS (Kalman and Lublin 1999). In a random sampling of 48 relapsing-MS (RMS) patients the HLA haplotype alleles proved to be quite diverse. As was true in other reports, HLA-DRB1*1501, HLA-DQB1*0602, and HLA-DQA1*0102 were evident and DRB1*1501 was the more prominent (Waid et al. 2014). However, DRB1*0404 and DRB1*0403 that typically associate with T1DM were found in numerous MS subjects (Waid et al. 2014). Th40 levels were elevated in RMS subjects regardless of HLA haplotype and as in T1DM, MS subjects carrying HLA alleles other than those associated with MS still had elevated Th40 cell numbers (Waid et al. 2014). As in T1DM, HC subjects that carried HLA-DRB1*1501, HLA-DQB1*0602, and HLA-DQA1*0102 alleles but did not present with MS had normal Th40 cell numbers. Pathogenicity of Th40 cells from MS subjects was assessed by classic antigen recall experiments. A subset of Th40 cells from RMS subjects recognized and responded to CNS-associated antigens in a classic antigen recall assay while Th40 cells from HC did not respond (Waid et al. 2014). Th40 cells from a small group of RMS subjects also responded to isolated human islets. Islets are encompassed by Schwann cells that are attacked during diabetogenesis (Winer et al. 2003). To address mechanism of action of Th40 cells during MS, reverse translation experiments using the experimental autoimmune encephalomyelitis (EAE) model were done (Vaitaitis et al. 2017b, 2019a).

Once initiated, the EAE model progresses similarly to human MS. An immune response directed to CNS-derived antigens creates lesions in the brain and spinal cord that rapidly progress to severe outcomes (Hertzenberg et al. 2013; Hussain et al. 2014). The protocol for EAE disease induction involves challenge with CNS-derived antigens, myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), or proteolipoprotein (PLP). Strong inflammatory conditions are established by complete Freund's adjuvant (CFA) composed of mycobacterial products suspended in mineral oil. Mice given MOG challenge with incomplete Freund's adjuvant (IFA, mineral oil with no inflammation inducing agents) do not develop disease (Visser et al. 2005). An important mechanism was induced expansion of Th40 cell numbers in the periphery (Vaitaitis et al. 2017b). CFA also primes innate dendritic cells through the toll-like receptors (TLR) (Visser et al. 2005). At 3 days post-CFA administration, Th40 cells expand from control levels to pathogenic levels, equivalent to levels observed in active disease RMS patients (Vaitaitis et al.

2017b, 2019a; Waid et al. 2014). Th40 cell numbers remain at pathogenic levels throughout the disease course. Longitudinal studies, over 8 years, of MS patients show sustained, elevated Th40 cell numbers in the PBMC. In EAE, symptoms arise at day 11 and progress from mild to severe within 2 weeks. At necropsy brains and spinal cords are infiltrated by Th40 cells (Vaitaitis et al. 2017b, 2019a). When Th40 cells were isolated from stage 3, early severe symptom mice, and transferred to SCID recipients (complete lack of lymphocytes), severe stage 4 symptoms could be induced with a single CFA treatment. Therapeutic approaches using antibodies directed against CD154, the CD40 receptor, prevent EAE induction and improve EAE preclinical scores (Howard et al. 1999, 2002a, b), suggesting that therapeutic target of Th40 cells would be beneficial.

Correlating the immune inflammation in autoimmune disease: Immune cells + HLA + chemokines + chemokine receptors: Both T1DM and MS, as discussed, represent how specific immune cells contribute to unique pathology outcomes. By focusing on a specific helper T cell, commonality between autoimmune diseases is discovered. Th1, Th2, and Th17 cells each are defined by the cytokines that they produce and secrete. There are no cell surface markers that differentiate them. Th1 and Th17 cells each contribute to autoimmune disease development, however there are studies showing that in vivo Th17 cells are precursor to Th1 (Bending et al. 2009; Scholzen et al. 2009; Cooke 2006). There always has been difficulty isolating Th17 cells from active disease in humans. Th17 cells were only described after in vitro generation; naïve $CD4^+$ cells treated with anti-CD3 + TGFβ (creating regulatory T cells) that then are treated with IL-6 and anti-IL-4. This protocol cannot occur in vivo (Kimura and Kishimoto 2010; Weaver et al. 2006). $CD4^+$ cells that express CD40 bridge the gap between Th1 and Th17 (Vaitaitis et al. 2017a, b; Waid et al. 2014). In preclinical mouse models of T1DM and MS, Th40 cell expansions in the periphery predate symptoms (Vaitaitis et al. 2017a, b). In human T1DM Th40 cell number expansions are universal and are elevated to pathogenic numbers in a subset of pre-T1DM, a portion of whom have gone on to develop T1DM (Vaitaitis et al. 2019b; Waid et al. 2007; Siebert et al. 2008). Th40 cell number expansions occur in MS (Waid et al. 2014). This expansion occurs in the early form of MS, clinically isolated syndrome, and in the progressive courses, SPMS and PPMS (observations and submitted manuscript, 2021).

Given the overall difficulty locating specific autoimmune disease biomarkers a combinational approach may be required. Th40 cell expansions indicate systemic inflammation. The elevated numbers in autoimmune disease are significantly higher than seen during infections (Waid et al. 2007, 2014). Infectious inflammation is temporary and once resolved, Th40 cell numbers recede. Th40 cell numbers in HC range from 13% to 25%; infectious disease range is 28–38%; T1DM range is 42–58%; and MS range is 34–90%. The lower Th40 numbers in MS reflects use of disease modulating therapies that target immune cells. Because HLA genes correlate with certain aspects of autoimmunity, they could be used as an indicator. However, clear drawbacks exist, HLA haplotypes are not diagnostic as discussed above. There are overlaps in HLA haplotypes between diseases (Waid et al. 2007, 2014). Also, there are subjects who develop disease without any of the "appropriate" HLA molecules.

Another consideration is chemokine and chemokine receptor expression. Chemokines are secreted molecules that direct immune cells to a specific inflammation site. At a site of infection/tissue damage/inflammation chemokines are secreted by resident innate immune cells, endothelium and epithelium. Immune cells including Th40 cells from local lymph nodes respond to the chemokine trail migrating to the site of damage. Chemokines associated with MS include CXCL12 and CXCL13 (Huber and Irani 2015; Krumbholz et al. 2006), and the interferon inducible chemokine IP-10 (Balashov et al. 1999). Chemokine receptors associated with MS include CCR2, CCR5 (Sato et al. 2012), CXCR3 (Cunill et al. 2018), CXCR4, and CXCR7 (Chu et al. 2017). Chemokine receptors associated with T1DM include CXCR5 (Liu et al. 2019), CXCR4, and CXCR3 (Waid et al. 2007; Chu et al. 2015). There are overlaps in chemokine receptor expressions between MS and T1 DM, but there are unique receptors as well.

Summary*:* Each autoimmune disease is distinct. There are, however, common features when specific immune components are considered. Many diseases have identified autoantibodies. In T1DM four AAbs have been identified and in subjects 5 years of age or younger there is strong disease predictive correlation with AAbs while in older subjects the rigor fails (Krischer et al. 2021). In MS, IgG oligocloncal bands are in the cerebrospinal fluid, but specificity is toward viruses and cell death–associated proteins (Giovannoni 2014). Thus, AAbs seem to indicate tissue damage, but do not correlate further to autoimmune disease development. Some autoimmunities, RA, and lupus, for example, have oxidative and nitric stress elements generated by activated macrophages, yet an associated biomarker has not arisen. Pathogenic T cells are common to all autoimmune diseases. Th1 and Th17 cells were indicated as major inflammation mediators. Until the discovery of CD40 expression on Th1 and Th17 cells, no common cell surface biomarker existed. Th40 cells are present at significantly ($p < 0.001$) elevated numbers in autoimmune diseases and are proving to be a peripheral blood biomarker for systemic inflammation. Th40 cell number expansions in preclinical mice models and in some pre-autoimmune categories of T1DM and MS indicate this cell type expansion occurs early. Because Th40 cells have an expanded TCR repertoire, their tissue targets are varied. In a subgroup of subjects, Th40 cells become expanded in number and remain expanded indefinitely; clear evidence of the pathogenicity of Th40 cells has been established (Vaitaitis et al. 2010, 2013, 2014, 2017a, b, 2019a, b; Wagner 2017; Waid et al. 2014; Vaitaitis and Wagner 2008, 2013). CD40 on Th40 cells preferentially induces inflammatory cytokines while CD28 preferentially induces regulatory cytokines (Vaitaitis et al. 2014, 2017a, b, 2019b, Waid et al. 2014). This crucial observation has been overlooked for many years. Previously all cytokine production was attributed to CD28.

Another feature of Th40 cells is TCR revision, which has distinct positive and negative attributes. TCR revision during a viral infection expands the TCR repertoire at a localized infection site, which aides in eliminating an infection more rapidly. In addition, revision provides downstream protection against future viral infections. The downside includes potential for developing self-tissue reactive T cells. In most subjects these autoaggressive T cells are eliminated by additional revisions or by

other regulatory control mechanisms. In some subjects regulatory mechanisms are faulty, resulting in persistent autoaggressive T cells. The search for specific viruses as causal in autoimmune disease has not held statistical rigor. The idea may be correct but in a more general sense. In autoimmune disease–prone subjects (all the features of autoimmunity clearly are yet to be discovered) multiple viral exposures lead to multiple TCR revisions, some of which are autoaggressive. In the absence of appropriate peripheral regulatory mechanisms these self-reactive T cells expand in number and lead to tissue destruction, e.g., autoimmunity. This hypothesis would account for the growing number of viruses, now including SARS-CoV-2, that contribute to autoimmune disease.

Mini Dictionary of Terms

- *Type 1 diabetes mellitus:* T1DM is a classic autoimmune disease. Immune cells including T and B cells macrophages and dendritic cells invade the islets of the pancreas leading to loss of insulin production. Systemic inflammation creates numerous clinical problems. There are no treatments.
- *Multiple sclerosis:* MS is a neurological disease that also is autoimmune. Immune cells invade the central nervous system leading to multiple clinical outcomes ranging in severity. The disease occurs in multiple courses beginning as a chronic disease that has remissions, but ultimately becomes progressive leading to more severe outcomes.
- *TCR revision*: It is the process by which mature T cells alter T cell receptor expression. The TCR provides the T cell with antigen specificity. When CD40 is engaged on Th40 cells under appropriate conditions, signals are delivered to induce changes in TCR expression. Depending upon the configuration of the TCR determines how the T cell will respond to an antigen. TCR revision has positive and negative consequences.
- *Th40 cells*: Th40 cells are created when the CD40 receptor is induced on CD4+ helper T cells. Inflammatory conditions during an infection or during autoimmunity create Th40 cells. CD40 on T cells induces inflammatory cytokines while CD28 on T cells induces regulatory cytokines.
- *Autoantibody*: It is an immunoglobulin molecule that can bind to a self-antigen. Antibodies normally target pathogens like viruses, but under autoimmune disease conditions, antibodies develop that target various self-tissues. Autoantibodies are a marker for tissue damage.

Key Facts

- Type 1 diabetes mellitus affects 1.25 million Americans and with the incidence increasing by 200,000 yearly. The incidence varies worldwide with an incidence of 0.1/100,000 in China and Venezuela and 36.8/100,000 in Sardinia and France.

- Multiple sclerosis affects almost 2.5 million worldwide and incidence is increasing by 200,000 yearly. Like diabetes disease incidence occurs in hot spots with higher incidence closer to the global poles and lower incidence at the equator. The reason is unknown.
- Th40 cells are a biomarker common to both diseases. Because of T cell receptor expression differences, the cells specifically attack different tissues. The increase in Th40 cells represent the systemic inflammation of each disease.
- There are no cures for T1DM or MS and while current treatment options target various immune components, not CD40 however, the diseases remain prevalent and progressive.

Summary Points

- This chapter focuses on defining a common biomarker in type 1 diabetes mellitus (T1DM) and multiple sclerosis (MS).
- Th40 cells develop in the periphery, have a diverse T cell receptor (TCR) repertoire, and under normal conditions their numbers remain low.
- During infection Th40 cell numbers increase but reduce in number over time; however, during systemic autoimmune inflammation Th40 cell numbers increase dramatically and do not reduce in number over time.
- CD40 on Th40 cells drives inflammatory cytokine production, while CD28 drives regulatory cytokines.
- Current biomarkers for T1DM and MS, HLA haplotype, genes identified by GWAS, and tissue-associated proteins do not hold diagnostic power.
- Viral infections increase peripheral Th40 cell numbers.
- Th40 cells can undergo TCR revision, alteration of the existing T cell receptor (TCR); this process increases the diversity of T cells responding to an infection and can account for the development of T cells able to respond to future infections.
- TCR revision can increase the risk of developing self-antigen reactive T cells that can promote autoimmune disease.

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Epigenetically Modified DNA Fragments

28

What They Are and How They Can Be Used as Biomarkers of Diabetes Pathogenesis and Risk

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Contents

Introduction 598
Epigenetic Modifications 598
Cell-Free DNA and Epigenetically Modified DNA Fragments 599
Technologies for DNA Methylation Detection 600
Differentially Methylated cfDNA as a Biomarker for β-Cell Death 601
Advancing Technologies to Measure Differentially Methylated cfDNA 603
Differentially Methylated cfDNA Beyond T1D 605
Limitations to Measuring Differentially Methylated cfDNA as a Biomarker for β-Cell Death 606
Applications to Prognosis, Other Diseases, or Conditions 607
Mini-Dictionary of Terms 609
Key Facts of Diabetes 609
Key Facts of Cell-Free DNA (cfDNA) 609
Summary Points 609
References 610

Abstract

Islet β-cell death is a hallmark of both type 1 and type 2 diabetes, resulting in decreased insulin levels. In recent years, circulating cell-free epigenetically modified DNA fragments arising from β-cells have been used to measure β-cell death as a biomarker for diabetes development and progression. Here, we review epigenetically modified DNA fragments, technologies to measure them, and usage of the tools to evaluate β-cell death in type 1 diabetes, type 2 diabetes, gestational diabetes, and islet cell transplantation.

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Keywords

Epigenetics · Cell-free DNA · Methylation · β-Cell death · Type 1 diabetes · Type 2 diabetes · Gestational diabetes · Islet transplantation · Insulin · Digital PCR · Methylation-specific PCR · Bisulfite conversion

Abbreviations

cfDNA	cell-free DNA
dPCR	digital polymerase chain reaction
lncRNA	long noncoding RNA
miRNA	microRNA
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
qPCR	quantitative polymerase chain reaction
T1D	Type 1 diabetes
T2D	Type 2 diabetes

Introduction

Both type 1 diabetes (T1D) and type 2 diabetes (T2D) are characterized by progressive β-cell failure. In T1D, this is typically caused by an autoimmune assault against the β-cells. By the time of diagnosis, β-cell mass is reduced by 70–80%; however, this loss occurs slowly over years, long before diagnosis with evidence of β-cell apoptosis (Marhfour et al. 2012; Campbell-Thompson et al. 2016). It has also been shown that individuals with T2D also have a significant reduction in β-cell mass with an increase in β-cell apoptosis (Sakuraba et al. 2002; Kahn 2003; Butler et al. 2003).

Through various mechanisms of cell death, predominately thought to be due to apoptosis, cellular DNA is shed into the bloodstream and other body fluids as cell-free DNA (cfDNA) (Jahr et al. 2001). As circulating cfDNA originates from dying cells, it thus reflects ongoing cell death taking place in the body (Wan et al. 2017). This concept, combined with the fact that epigenetic modifications are tissue specific, led to the concept of tracking tissue-specific cellular death through the methylation status of specific genes found in cfDNA. The use of epigenetic marks has revolutionized the field of noninvasive molecular diagnosis (Gai and Sun 2019; Rahat et al. 2020). This chapter will focus on the use of epigenetic marks, specifically DNA methylation, in cfDNA during the pathogenesis of diabetes as a measure of β-cell death.

Epigenetic Modifications

Epigenetics is the study of how cells control gene activity without changing the DNA sequence. Chemical modifications of DNA alter DNA accessibility and chromatin structure and thereby regulate or alter gene transcription rates. Chemical

modifications are highly variable among different individuals, in different tissues within an individual, and within different cells within a given tissue. There are three main types of epigenetic modifications that impact gene expression: DNA methylation, histone modification (methylation, acetylation, phosphorylation), and noncoding RNAs.

DNA methylation refers to the addition of a methyl group ($-CH_3$), typically to the fifth carbon atom of a cytosine ring. The conversion of a cytosine base to a 5-methylcytosine is catalyzed by DNA methyltransferases. Generally, this happens to cytosine residues that lie next to a guanine base resulting in what is known as CpG methylation. DNA methylation results in suppression of gene transcription in most cases. The methylation status of specific genes is very tissue and cell-type specific (Moss et al. 2018).

Histones are proteins that condense and package DNA into nucleosomes. Modifications to the histones impact gene transcription, chromosome packaging, DNA damage, and DNA repair (Bannister and Kouzarides 2011). Histone acetylation is mostly associated with a chromatin structure that is open and therefore accessible to transcription factors, increasing gene transcription. Histone methylation is the transfer from S-adenosyl-L-methionine of one to three methyl groups to lysine or arginine residues of histone proteins. Histone methylation is associated with both activation and repression of transcription. Histone phosphorylation occurs when histone kinases take phosphate groups from ATP and add them to the hydroxyl group of target amino-acid side chains. Histone phosphorylation is important for chromosome condensation during cell division, DNA repair, and transcription regulation.

Noncoding RNAs are a cluster of RNAs that do not encode functional proteins (Wei et al. 2017). MicroRNAs (miRNAs) interact primarily with the 3′ untranslated region of target messenger RNAs (mRNA) to induce mRNA degradation and translational repression; however, they have also been shown to interact with the 5′ untranslation region, coding sequences, and gene promoters. Under specific conditions, miRNAs can activate translation or regulate transcription (O'Brien et al. 2018). Long noncoding RNAs (lncRNAs) are transcripts that are longer than 200 base pairs and modulate gene expression and function through epigenetic regulation of chromatin structure. lncRNAs also regulate posttranscriptional mechanism including transcript splicing, mRNA decay, and protein translation. Unlike miRNAs, lncRNAs are unique in that their function does not require specific sequences (Yeh et al. 2020).

Cell-Free DNA and Epigenetically Modified DNA Fragments

In 1948, Mandel and Metais first detected the presence of cell-free nucleic acids in human blood (Mandel and Metais 1948). In 1977, elevated circulating cfDNA levels in patients with cancer were first reported (Leon et al. 1977). cfDNA is released from cells through apoptosis, necrosis, and active secretion (Jahr et al. 2001; Thierry et al. 2016). In healthy individuals, cfDNA is mainly attributed to lymphoid and myeloid tissues (Snyder et al. 2016), while in various physiological/pathological conditions,

the associated or affected tissues would release additional cfDNAs into the blood resulting in elevated cfDNA (Mok 1971; Rodrigues Filho et al. 2014; De Vlaminck et al. 2015). In 1989, Stroun et al. reported that some of the cfDNA in cancer patients originated from cancer cells (Stroun et al. 1989), and in 1994, the first mutated cfDNA was identified in plasma from cancer patients (Sorenson et al. 1994).

Circulating cfDNA molecules are highly degradable and predominantly occur as small size (roughly 100–200 base pairs) as double-stranded DNA (Grabuschnig et al. 2020; Heitzer et al. 2020). The half-life of circulating cfDNA is between 16 mins and 2.5 h (Chused et al. 1972; Tamkovich et al. 2006; Diehl et al. 2008). Circulating cfDNA retains its epigenetic marks, allowing for the detection of tissue-specific DNA fragments (Snyder et al. 2016; Lehmann-Werman et al. 2016). Notably, methylation of specific regions of DNA is highly conserved in specific tissue and cell types (Song et al. 2009). In 1999, Wong et al. described a pattern of differentially methylated cfDNA in the plasma of cancer patients of the p16 gene (Wong et al. 1999), leading to the first use of differentially methylated cfDNA as a potential biomarker. In 2011, Akirav et al. reported the first use of a differentially methylated cfDNA assay to look at β-cell death (Akirav et al. 2011) (Table 1).

Technologies for DNA Methylation Detection

Bisulfite conversion-based methods are the current "gold standards" for DNA methylation studies. Treatment of denatured DNA with sodium bisulfite leads to deamination of the unmethylated cytosines into uracils, while the methylated cytosines remain unaffected (Fig. 1) (Frommer et al. 1992). The bisulfite-converted DNA can then be used in PCR or sequencing assays to determine the initial methylation status of the DNA template.

For measurement of known differentially methylated targets after bisulfite conversion, there are several different PCR- and sequencing-based methods available (Kurdyukov and Bullock 2016). Methylation-specific PCR uses two distinct methylation-specific primer sets for detecting the DNA of interest. The first primer is methylation-specific and will amplify bisulfite-converted methylated DNA and untreated DNA, while the second primer is unmethylation-specific and will amplify bisulfite-converted unmethylated DNA (Herman et al. 1996). Methylation-specific PCR has been combined with high-resolution melting as DNA that was originally methylated retains the cytosine residues that have a higher melting point, and therefore the level of methylation is correlated with a higher melting profile (Wojdacz et al. 2008). Multiplexing probe-based quantitative PCR (qPCR) allows for using methylation-unspecific primers along with methylation-specific probes of different fluorescent properties (typically FAM and VIC) to allow for the measurement of both unmethylated and methylated DNAs using the same primers. Digital PCR has further advanced the specificity and sensitivity of methylation-specific PCR by allowing for absolute quantification through partitioning the reaction (Mao et al. 2019). Bisulfite-converted DNA can also be used for the amplification of the region of interest followed by sequencing. Another technology that has been successful for

Table 1 Published studies using differentially methylated cfDNA to measure β-cell death

Study	Technique	Gene	Disease
(Akirav et al. 2011)	qPCR	*INS*	T1D
(Husseiny et al. 2012)	qPCR	*INS*	T1D
(Lebastchi et al. 2013)	qPCR	*INS*	T1D
(Fisher et al. 2013)	qPCR	*INS*	T1D
(Husseiny et al. 2014)	qPCR	*INS*	T1D Islet transplantation
(Usmani-Brown et al. 2014)	dPCR	*INS*	T1D
(Herold et al. 2015)	dPCR	*INS*	T1D
(Fisher et al. 2015)	dPCR	*INS*	T1D
(Lehmann-Werman et al. 2016)	DNA seq	*INS*	T1D Islet transplantation
(Olsen et al. 2016)	qPCR	*IAPP*	T1D
(Tersey et al. 2016)	dPCR	*INS*	T1D
(Kenna et al. 2016)	qPCR	*INS*	Gestational diabetes
(Bellin et al. 2017)	dPCR	*INS*	Islet transplantation
(Sklenarova et al. 2017)	dPCR	*GCK, INS*	T1D
(Tersey et al. 2018)	dPCR	*INS*	T2D
(Mulukutla et al. 2018)	dPCR	*INS*	Ketosis-prone diabetes
(Roels et al. 2019)	dPCR	*INS*	Islet transplantation
(Farr et al. 2019)	dPCR	*INS*	T1D
(Neyman et al. 2019)	dPCR	*INS*	T1D
(Simmons et al. 2019)	dPCR	*INS*	T1D
(Speake et al. 2020)	dPCR DNA seq	*INS*	Islet transplantation
(Neiman et al. 2020)	DNA seq	*INS*	T1D Islet transplantation
(Syed et al. 2020)	dPCR	*INS, CHTOP*	T1D T2D
(Arosemena et al. 2021)	dPCR	*INS*	T2D
(Gitelman et al. 2021)	dPCR	*INS*	T1D

low-throughput assays is pyrosequencing. The level of methylation for each CpG site within the sequenced region is estimated based on the signal intensities for incorporated dGTP and dATP resulting in quantitative data (Tost and Gut 2007).

Differentially Methylated cfDNA as a Biomarker for β-Cell Death

Based on techniques and approaches developed for measuring differentially methylated cfDNA in cancer and pregnancy, it was proposed that DNA species arising from dying β-cells could be measured in the circulation of individuals with ongoing β-cell death (Fig. 2). As insulin is almost exclusively expressed in islet β-cells, the gene encoding preproinsulin (*INS* in humans) is largely unmethylated in β-cells and

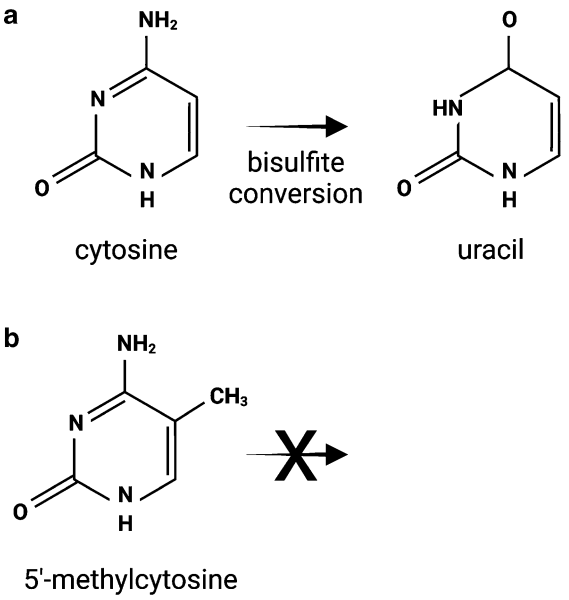

Fig. 1 Diagram of bisulfite conversion. (**a**) Unmethylated cytosines are converted to uracils; (**b**) methylated cytosines remain as cytosines. (Created with Biorender)

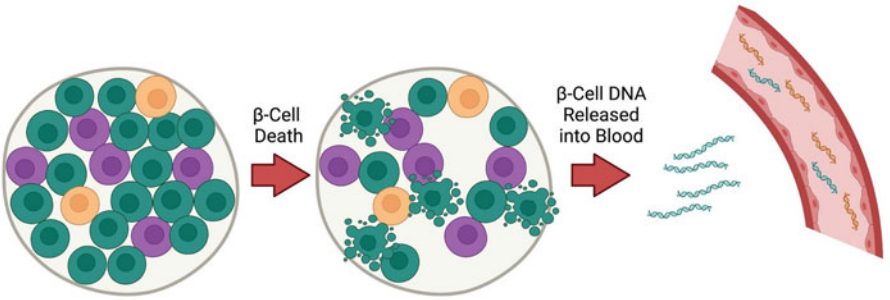

Fig. 2 β-Cell-specific cell-free DNA. Upon β-cell death, fragmented DNA with β-cell-specific epigenetic marks is released into the bloodstream. (Created with Biorender)

methylated in all other cell types (Kuroda et al. 2009). Akirav and colleagues developed the first assay to measure unmethylated *INS* DNA using a nested PCR procedure followed by SYBR-green qPCR using methylation-specific primers to measure the quantity of unmethylated versus methylated *Ins1* in mouse cell lines and diabetic mouse models, as well as unmethylated versus methylated *INS* in plasma samples from individuals with recent-onset T1D (Akirav et al. 2011). Akirav and colleagues found that DNA derived from β-cells was 60–80% unmethylated at seven different CpG locations on the mouse *Ins1* gene compared to 10–30% unmethylated

in liver cells. Next, they used two different models of diabetes in mice. In the first model, a high-dose streptozotocin injection induces rapid β-cell death. They found that β-cell death, as measured using a ratio of unmethylated-methylated *Ins1* DNA, was increased at both 8 and 24 h after injection, prior to the increase of blood glucose at 24 h after injection. The second mouse model they used is the NOD mouse, which develops spontaneous diabetes between 12 and 25 weeks of age and exhibits β-cell stress as early as 6 weeks of age (Tersey et al. 2012). The authors found that the unmethylated-methylated *Ins1* DNA ratio was increased at 14 weeks of age prior to increased blood glucose levels. Next, they compared cfDNA isolated from serum from control individuals and individuals diagnosed with T1D within 1 year and found an increased ratio of unmethylated-methylated *INS* DNA in the individuals with new-onset T1D (Akirav et al. 2011). This work stimulated the future development of new techniques to measure β-cell death using differentially methylated cfDNA.

Shortly after the introduction of using differentially methylated cfDNA, the first study using it as a marker for β-cell death in a clinical trial was published in 2013. Lebastchi et al. showed that while placebo- and teplizumab-treated subjects had a similar level of unmethylated *INS* DNA at baseline, the teplizumab-treated subjects but not the placebo-treated subjects had a decreased level of unmethylated *INS* DNA at the 1-year endpoint (Lebastchi et al. 2013), suggesting reduced β-cell death after teplizumab treatment. These data suggest that it may be possible to use a β-cell death biomarker as a treatment outcome to study the use of diabetes therapeutics; however, this potential use has not been studied yet.

In 2015, Herold et al. showed that individuals with high risk of T1D, as determined by having dysglycemia and two autoantibodies present, had significantly elevated unmethylated-methylated *INS* cfDNA ratio compared to individuals with no risk or individuals with T1D (Herold et al. 2015). They also showed that individuals that progressed to developing diabetes within a 3–4-year follow-up had increased β-cell death as measured by unmethylated-methylated *INS* cfDNA. Syed et al. found that autoantibody-negative first-degree relatives have increased unmethylated and methylated *INS* cfDNA suggesting β-cell death maybe genetic and occurs long before the presence of diabetes (Syed et al. 2020) (Fig. 3). These data suggest that a β-cell death biomarker might be utilized to determine which individuals may be at risk for T1D.

Advancing Technologies to Measure Differentially Methylated cfDNA

Up until this point, prior methods used SYBR-Green PCR methodologies to correlate the appearance of unmethylated human *INS* or mouse *Ins1/2* cfDNA to dying β-cells. SYBR-Green PCR methodologies have several limitations, including high background signals and the need for several primer combinations and repeated PCR assays to assess multiple DNA targets in a single sample. Fisher et al. first described and used a dual fluorescent probe-based multiplex PCR approach to measure both

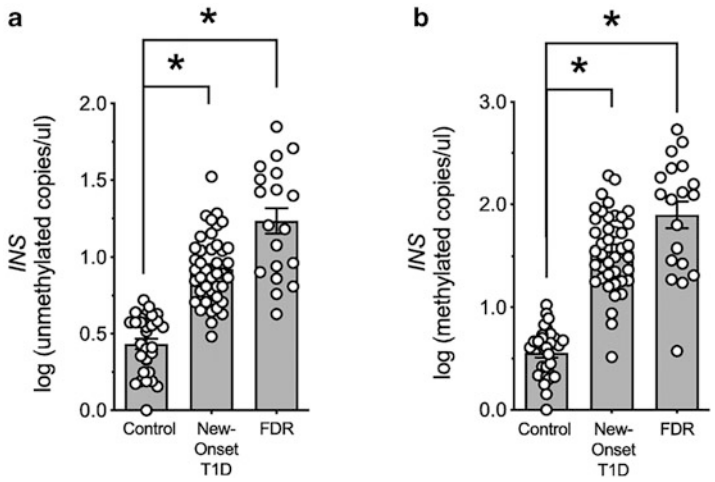

Fig. 3 β-Cell death in youth with T1D. cfDNA was isolated from serum obtained from normal unrelated control youth, youth with new-onset T1D, and autoantibody-negative first-degree relative (FDR) youth. (**a**) Unmethylated *INS* DNA; (**b**) methylated *INS* DNA. (Graphs are modified from Syed et al. (2020) under the open-access Creative Common BY license)

unmethylated and methylated *Ins2* cfDNAs in a single PCR reaction and eliminated the need to run multiple PCRs for comparison and increased sensitivity (Fisher et al. 2013) (Fig. 4). Usmani-Brown et al. moved the technology another step forward increasing sensitivity even further with the introduction of digital PCR (Usmani-Brown et al. 2014). Digital PCR allows for absolute quantification through partitioning the reaction into thousands of reaction samples (droplets), significantly increasing the sensitivity of the reaction from a single sample (Farr et al. 2019). Lehman-Werman et al. developed a new methodology using sequencing to identify more CpG sites and increased specificity of their assay over others (Lehmann-Werman et al. 2016).

In addition to advancing the PCR technologies, studies have also improved upon the methodology by using different CpG sites. Olsen et al. hypothesized that *IAPP* (amylin) would be specifically unmethylated in β-cells as it is expressed predominately in β-cells and released alongside insulin. They discovered several CpG sites that were differentially unmethylated in β-cells and designed primers that sat over five different sites in mouse and two different sites in humans compared to only one different site in past studies. Using these *IAPP* primers, they found increased β-cell death in individuals with recent-onset T1D compared to control individuals (Olsen et al. 2016). Along the same lines, Sklenarova et al. hypothesized and discovered β-cell-specific unmethylated CpG sites in the *GCK* (glucokinase) gene. However, when used in clinical samples, they found no differences among unmethylated *GCK* DNA in their samples from individuals with diabetes or prediabetes (autoantibody positive), but found a significant increase in methylated *GCK* DNA in the individuals with T1D (Sklenarova et al. 2017).

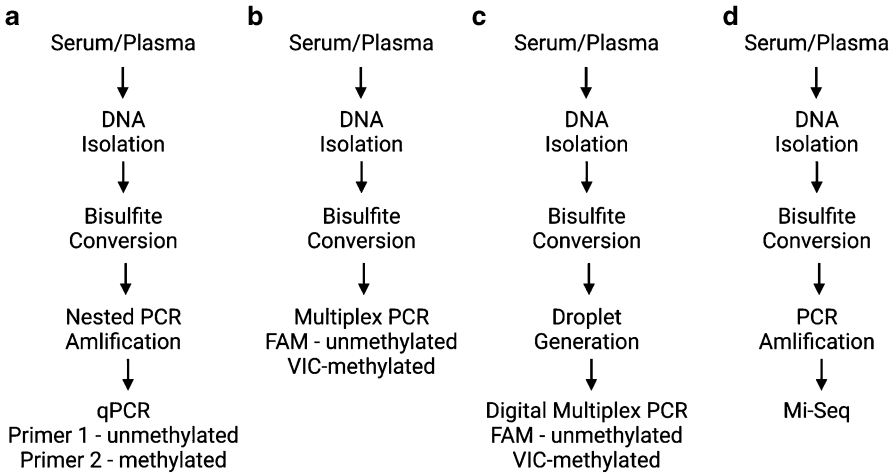

Fig. 4 Assays to measure differentially methylated cfDNA. (**a**) qPCR after nested PCR; (**b**) multiplex qPCR; (**c**) digital multiplex PCR; (**d**) methyl-DNA-seq. (Created with Biorender)

The aforementioned studies examined genes known to be transcribed into proteins that are almost exclusively expressed by β-cells. Syed et al. used an unbiased approach and assessed DNA methylation from 64 human islet preparations and leveraged data from 27 publicly available non-islet human tissues and identified 6201 differentially methylated sites specific to human islets (Syed et al. 2020). They went on to further examine 24 different sites across 5 different genes and found *CHTOP* to be the most uniformly unmethylated in β-cells compared to other tissue types. Interestingly, CHTOP is not exclusively expressed in β-cells. Both unmethylated and methylated *INS*, but only methylated *CHTOP* DNA, were elevated in individuals with new-onset T1D as well as autoantibody-negative first-degree relatives and proposed that the increased methylated DNA is due to increased inflammation which they also found to be increased in individuals with sepsis (Syed et al. 2020).

Differentially Methylated cfDNA Beyond T1D

While most of the published studies have focused on using differentially methylated cfDNA to measure β-cell death during the pathogenesis of T1D, some have used the developed assays during other conditions of β-cell death. During islet transplantation, it is expected that the transplanted islets have a significant fraction of lysed β-cells and that the immediate posttransplant period exhibits instant blood-mediated inflammatory reaction to islet death (Bennet et al. 2000). Fisher et al. used a mouse model of human islet xenotransplantation (into mice) and showed that unmethylated human *INS* DNA was released immediately into the circulation (Fisher et al. 2015). It has also been shown that the levels of unmethylated *INS* DNA increased acutely

following islet transplantation and that this signal subsides within hours to days following transplantation (Husseiny et al. 2014; Bellin et al. 2017; Roels et al. 2019; Speake et al. 2020; Neiman et al. 2020).

T2D develops secondary to insufficient insulin secretion in the context of insulin resistance in peripheral tissues; however, clinical manifestations of the disease occur only after substantial loss of functional β-cell mass. Tersey et al. first examined β-cell death in T2D by cell-free DNA using a mouse model of diet-induced obesity and dysglycemia (Tersey et al. 2018). While these mice do not develop frank diabetes, they are a good model of prediabetes. They found that β-cell death was episodic during the development of obesity and glucose intolerance with an increase in unmethylated *Ins2* cfNA. Similarly, Syed et al. observed that in obese youth, there is an increase in β-cell death with an increase in unmethylated *INS* and *CHTOP* cfDNA; however, when the obese youth cohort was stratified into groups with differing glycemic control, there was no difference among these cross-sectional cohorts, suggesting that the overall increase in unmethylated cfDNA species in the obese youth reflect differences largely driven by weight (Syed et al. 2020) (Fig. 4a, b). However, in obese adults, Arosemena et al. found no differences in unmethylated *INS* cfDNA compared to lean controls (Arosemena et al. 2021) (Fig. 4c, d). These data suggest that β-cell death is exhibited episodically during obesity and T2D progression, and longitudinal cohorts need to be studied.

Similar to T2D, in gestational diabetes, the ability of the β-cells to control glucose levels is impaired due to an overall insulin resistant, increased insulin demand, and impaired β-cell function. To determine if gestational diabetes was due to increased β-cell death, Kenna et al. used a differentially methylated *INS* cfDNA assay to measure β-cell death in women with gestational diabetes during the second and third trimesters and compared to nonpregnant women, pregnant women, and postpartum women with normal pregnancies (Kenna et al. 2016). They found that pregnancy alone slightly increased β-cell death and was significantly reduced in women with gestational diabetes compared to those with normal pregnancies. Postpartum levels were similar to nonpregnant levels (Kenna et al. 2016).

Limitations to Measuring Differentially Methylated cfDNA as a Biomarker for β-Cell Death

While accurate and sensitive detection of differentially methylated cfDNA is improving, it remains challenging to measure for a number of different reasons. Regardless of assay and disease status, the amount of β-cell DNA in circulation is extremely low. Additionally, the amount of blood that is/can be obtained from a single visit is limited, resulting in small yields of cfDNA after isolation. Also, cfDNA is vulnerable to contamination by the lysed blood cells if not processed soon after blood collection (Pös et al. 2020). Different types of cfDNA isolation protocols result in varying yields leading to significant variations in cfDNA (Sorber et al. 2017). Another major limitation to measuring methylated cfDNA is the short half-life. The half-life of cell-free DNA has been reported to be approximately 2 h

(Chused et al. 1972; Tamkovich et al. 2006; Diehl et al. 2008). This means that any given blood sample only has cfDNA from β-cells that have died very recently, and one cross-sectional sample is most likely not a good indication of what is happening during disease progression.

All of the current studies require bisulfite conversion of isolated circulating DNA prior to utilization in the assays. However, bisulfite conversion is known to cause the degradation of the input DNA, which results in loss of usable cfDNA (Grunau et al. 2001). Additionally, the increased guanine-cytosine content makes PCR primer design and sequencing more challenging (Gai and Sun 2019).

The majority of the current β-cell death assays use different methylated sites along the *INS* gene or promoter. A limitation to that is that these sites along the *INS* gene are only approximately 80–90% unmethylated in β-cell DNA (Syed et al. 2020). Several newer assays have started using different genes and using unbiased methods to find CpG sites that might show more specificity to the β-cell (Fig. 5).

Applications to Prognosis, Other Diseases, or Conditions

To date, circulating differentially methylated cfDNA is still in development as a tool for diabetes. In the case of T1D, differentially methylated cfDNA studies have identified evidence of β-cell death in the early phases of the disease and decreases of β-cell death after positive therapeutic intervention. This leads to the proposal that differentially methylated cfDNA assays have the potential to be used as both a biomarker for identifying individuals at high-risk of developing T1D and as an outcome biomarker of therapeutic benefit. In the case of T2D, it is still unclear if measurable β-cell death is occurring. More studies using longitudinal datasets in humans are required to determine the usefulness of differentially methylated cfDNA in the settings of obesity, dysglycemia, and T2D. For islet transplantation, studies have shown a significant increase in β-cell death directly after transplantation; however, it remains to be determined if these assays will be useful in determining the individual therapeutic benefit of a specific transplantation.

Measurement of differentially methylated cfDNA has been long been utilized in other diseases and conditions. Prior to its use in diabetes, differentially methylated cfDNA was used in predominately cancer patients and for fetal analysis in pregnant women. DNA methylation in combination with liquid biopsy is extremely powerful in identifying circulating epigenetic biomarkers of clinical importance. In cancer, the incidence of aberrant methylation of specific CpG islands is high in tumor samples and can thus be easily detected by using genome-wide screening technologies, in contrast to cancer-specific mutation, which are both rare and spread out among many different positions in tumor DNA (Lianidou 2016). However, the number of clinical tests that are commercially available is relatively low (Lianidou 2021). In fetal testing, differentially methylated specific cfDNA marks have shown promise in determining the sex of the fetus and risk for severe preeclampsia, preterm birth, and intrauterine growth restriction (Hui and Chiu 2016).

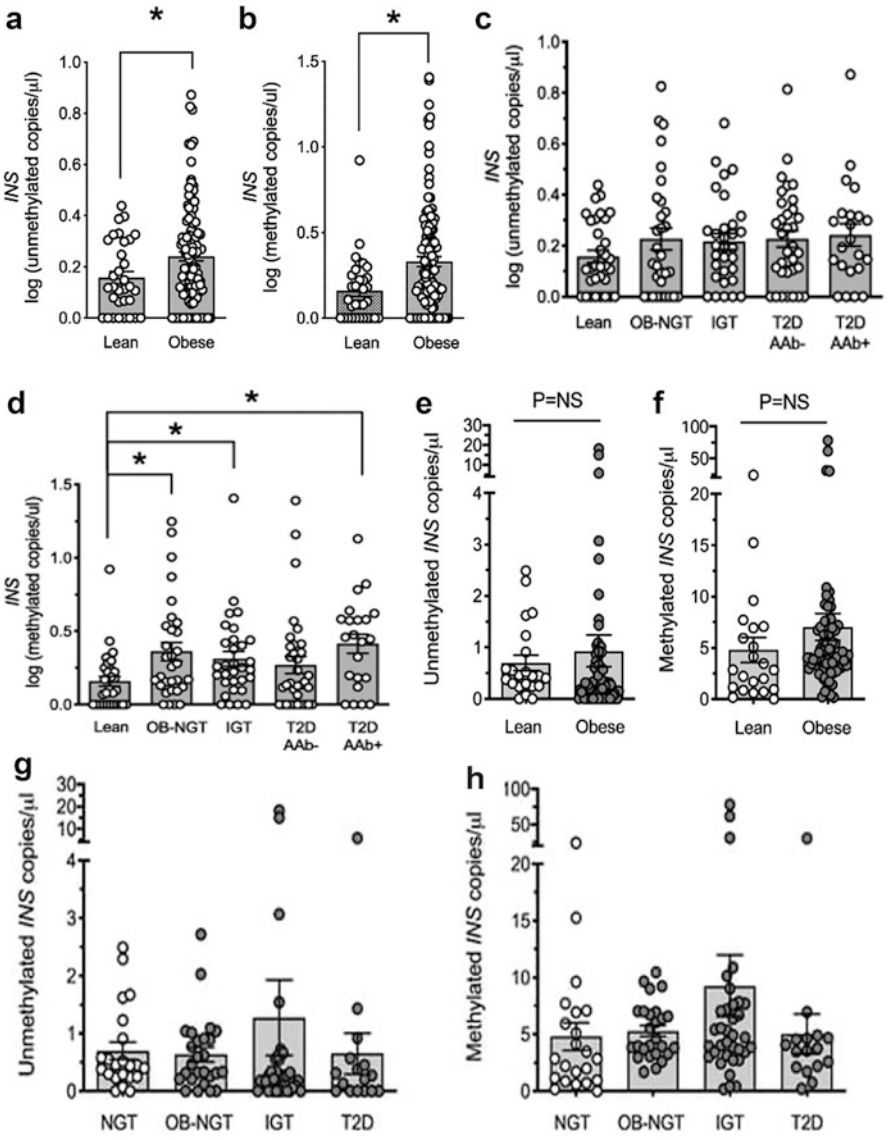

Fig. 5 β-Cell death in youth and adults with obesity and T2D. cfDNA was isolated from serum obtained from normal lean and obese youth and adults stratified further into cohorts based on glycemic control NGT (lean and normal glucose tolerance), OB-NGT (obese and normal glucose tolerance), IGT (impaired glucose tolerance), T2D AAb- (type 2 diabetes, autoantibody negative), and T2D AAb+ (type 2 diabetes, autoantibody positive). (**a**) Unmethylated *INS* DNA in youth; (**b**) methylated *INS* DNA in youth; (**c**) unmethylated *INS* DNA in stratified youth; (**d**) methylated *INS* DNA in stratified youth; (**e**) unmethylated *INS* DNA in adults; (**f**) methylated *INS* DNA in adults; (**g**) unmethylated *INS* DNA in stratified adults; (**h**) methylated *INS* DNA in stratified adults. **P* <0.05 for the comparisons indicated. (Graphs are modified from Syed et al. (2020) and Arosemena et al. (2021) under the open-access Creative Common BY license)

Mini-Dictionary of Terms

- **Bisulfite conversion**. Treatment of denatured DNA with sodium bisulfite leading to deamination of the unmethylated cytosine into uracil, while the methylated cytosine residues remain unaffected
- **Cell-free DNA (cfDNA)**. Fragmented cellular DNA that has been released into the circulation after apoptosis, necrosis, or active secretion
- **Epigenetics**. The study of how cells control gene activity without changing the DNA sequence
- **Methylation**. An epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine
- **β-cells**. An endocrine cell located within the islets of Langerhans within the pancreas that synthesis and secrete insulin

Key Facts of Diabetes

- *Over 422 million people have diabetes.*
- *Diabetes is the major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation.*
- *Type 1 diabetes results from the autoimmune destruction of β-cells.*
- *Type 2 diabetes results from impaired insulin resistance leading to β-cell dysfunction and death.*
- *β-Cell death occurs during the pathogenesis of both type 1 and type 2 diabetes.*

Key Facts of Cell-Free DNA (cfDNA)

- *cfDNA is primarily released from cell upon conditions of apoptosis, necrosis, and active secretion.*
- *cfDNA is highly degradable and have a short half-life of 16 mins–2 h.*
- *cfDNA is double stranded and on average 100–200 base pairs in length.*
- *cfDNA retains its epigenetic marks.*
- *cfDNA has been used to measure cell death from serum/plasma samples and as a biomarker for disease status.*

Summary Points

- Epigenetics is the study of how cells control gene activation and repression without changing the DNA sequence through chemical modifications of DNA to alter DNA accessibility and chromatic structure.
- Cellular DNA is released into the circulation upon cell death and maintains its epigenetic marks, allowing for the determination of cell-type-specific cfDNA as a marker of cell death.

- The insulin (*INS*) gene is approximately 80–90% unmethylated in β-cells and 10–20% unmethylated in all other cell types allowing for the measurement of β-cell death.
- Early studies have utilized methylation-specific *INS* DNA assays and reported elevations in unmethylated *INS* cfDNA in individuals with T1D, T2D, and after islet transplantation.
- The development of cfDNA as a biomarker for the risk of diabetes is still ongoing.

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Urinary Glucose Excretion as a Biomarker for Precision Medicine in Diabetes

29

Shinsuke Noso and Hiroshi Ikegami

Contents

Introduction 616
The Role of the Kidney in Glucose Homeostasis 617
Physiology of Glucosuria 617
Clinical Determinants of Urinary Glucose Excretion in the Diabetic State 618
Sodium-Glucose Cotransporters and Glucosuria 620
Mutations in the SGLT2 Gene and Urinary Glucose Excretion 621
Common Variants of the SGLT2 Gene 621
SLC5A2 and Diabetes-Related Phenotypes 622
Association of SGLT2 Gene with Susceptibility to Type 2 Diabetes Mellitus 623
Application to Prediction 625
SLC5A2 rs9934336 Polymorphism as a Genetic Marker of Diabetes-Related Phenotypes 625
Mini-Dictionary of Terms 625
Key Facts of Urinary Glucose Excretion 626
Summary Points 626
References 626

Abstract

Glucosuria has been recognized as a classical biomarker for poor glycemic control among individuals with diabetes. However, the development of sodium-glucose transport protein 2 (SGLT2) inhibitors has made diabetologists reconsider glucosuria as an alternative biomarker to achieve better glycemic control and prevent renal and cardiovascular outcomes. Since inter-individual variations are often observed in the amount of urinary glucose excretion and the efficacy of SGLT2 inhibitors, the determinants of urinary glucose excretion need to be

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analyzed to further understand this biomarker, leading to the establishment of precision medicine using SGLT2 inhibitors. In this chapter, the clinical and genetic determinants of urinary glucose excretion, including the SGLT2 gene encoded by *SLC5A2*, are summarized, and its clinical usefulness is discussed for precision medicine in diabetes.

Keywords

Glucosuria · Urinary glucose excretion · Diabetes mellitus · *SLC5A2* · Single nucleotide polymorphism · Association study · Meta-analysis · Multiple regression analysis · SGLT2 inhibitor · Blood glucose · eGFR · Sex

Abbreviations

AUC	area under the curve
BMI	body mass index
CGM	continuous glucose monitoring
eGFR	estimated glomerular filtration rate
HOMA-IR	homeostasis model assessment insulin resistance
SGLT	sodium-glucose transporter
SLC5A2	solute carrier 5A2
SNP	single nucleotide polymorphism

Introduction

Glucosuria is one of the most prominent symptoms of hyperglycemia and is a well-known noninvasive and convenient biomarker of diabetes mellitus with poor glycemic control. Hence, it is frequently used to screen for juvenile diabetes at school age for public health (Kim and Lee 2017; Urakami et al. 2005, 2006). In renal blood flow, glucose is excreted into the urine when the blood glucose level continuously increases above the threshold of glucose reabsorption in the renal proximal tubules. Although the average threshold is considered to be approximately 160–180 mg/dL, large inter-individual differences in the threshold and amount of urinary glucose excretion have been observed. In order to better manage blood glucose and prevent chronic complications associated with diabetes mellitus, the measurements of HbA1c and the self-measurement of blood glucose are considered as suitable biomarkers, particularly for individuals with blood glucose levels below the threshold of glucosuria. Therefore, the detection of glucose through urinalysis has not played a pivotal role in the management of blood glucose in diabetes mellitus in the past decades until the development of SGLT2 (sodium-glucose transporter 2) inhibitors as a novel pharmacological intervention. SGLTs are predominantly expressed in the proximal tubule of the kidney and transport glucose from the glomerular filtrate into the circulation, resulting in the elimination of glucosuria under normal conditions. Since the genetic mutations in the SGLT2 gene can cause familial renal glucosuria even with normal blood glucose levels, this gene could be an attractive candidate as a

genetic determinant for urinary glucose excretion and the development of diabetes mellitus. Accumulating evidence has revealed the contribution of the SGLT2 gene as an independent genetic factor to the inter-individual differences in urinary glucose excretion and its association with the susceptibility to type 2 diabetes. Thus, in this chapter, we summarize the clinical and genetic determinants of urinary glucose excretion as a biomarker for diabetes mellitus and highlight the genetic association of the SGLT2 gene with type 2 diabetes.

The Role of the Kidney in Glucose Homeostasis

The kidney plays a critical role in glucose homeostasis in both physiological and pathological conditions (Meyer et al. 2002). Glucose utilization and production contribute to glucose metabolism in the kidney, similar to other organs, such as the skeletal muscles and adipose tissues. The renal medulla is mainly responsible for glucose uptake from the circulation and utilization for energy requirements (Gerich et al. 2001). Relative to the total glucose utilized by the whole body, the kidney consumes approximately 5–10% in the fasting state and 10–15% in the postprandial state (Gerich 2010). Most of the energy in the kidney is spent reabsorbing glucose from the glomerular filtrate in the proximal convoluted tubule of the renal cortex, although free fatty acids, rather than glucose, are the main source of energy in the renal cortex. On the other hand, glucose is synthesized from lactate, glutamine, alanine, and glycerol in the renal cortex since it contains the key enzymes for gluconeogenesis, such as glucose 6-phosphatase, fructose 1,6-diphosphatase, and phosphoenolpyruvate carboxykinase (Gerich et al. 2001; Guder and Ross 1984). Renal glucose production accounts for approximately 40% of total glucose release in the whole body (Gerich et al. 2001), and this is known to increase in individuals with type 2 diabetes, contributing to the elevated blood glucose levels (Eid et al. 2006; Meyer et al. 1998).

In addition to the production and utilization of glucose, the kidney plays a notable and unique role in glucose homeostasis by returning glucose from the urine to the circulation. The reabsorption of glucose from the glomerular filtrate occurs in the proximal tubules of the renal cortex (Gamba et al. 1994; Gerich et al. 2001; Stumvoll et al. 1995).

Physiology of Glucosuria

Glucosuria refers to the physiological excretion of glucose into the urine due to its excessive levels in the glomerular filtrate (primary urine), which is beyond the threshold of glucose excretion at the kidney's proximal tubule (Cowart and Stachura 1990). In healthy individuals, approximately 180 g/day of glucose is filtered by the glomeruli of both kidneys. Then, the glucose in the primary urine is completely absorbed by the SGLTs in the proximal tubules, resulting in the non-detection of glucose in the urine under normal conditions. Owing to the noninvasive and

inexpensive nature of urinalysis, the measurement of glucosuria is highly versatile for the early medical detection of asymptomatic diabetes mellitus at schools and workplaces in several countries (Kim and Lee 2017; Urakami et al. 2005, 2006).

Although the average threshold of urinary glucose excretion is 160–180 mg/dL in healthy individuals, this is known to vary inter-individually, ranging from 140 to 200 mg/dL (Peters and Van Slyke 1946). The average threshold of urinary glucose excretion is higher in the diabetic state than in the physiological condition, leading to a reduced urinary glucose excretion and elevated blood glucose levels (Freitas et al. 2008; Mogensen 1971; Rahmoune et al. 2005). In addition to the increase in the average threshold of urinary glucose excretion in patients with diabetes mellitus, the inter-individual variation of the thresholds also markedly extends, ranging from 54 to 300 mg/dL of blood glucose (Griffin et al. 1979; Johansen et al. 1984; Lawrence 1940; Malone et al. 1976; Robertson and Gray 1953; Service et al. 1972; Walford et al. 1980). Various clinical factors, such as blood glucose level, age, and sex, can influence the thresholds of glucosuria in individuals with diabetes mellitus. The significance of each factor on glucosuria, however, should be analyzed by multivariate analysis after precisely measuring the urinary glucose excretion using the 24-h urine collection.

Clinical Determinants of Urinary Glucose Excretion in the Diabetic State

Among the various clinical factors that affect urinary glucose excretion, the blood glucose level is the strongest independent determinant in diabetes mellitus by multiple regression analysis (β, standard partial regression coefficient: 0.41) (Monobe et al. 2021). The higher the levels of blood glucose, the larger the amount of urinary glucose excretion in the diabetic state. Monobe et al. tested various indices related to blood glucose levels, such as HbA1c, fasting blood glucose, average blood glucose, and area under the curve (AUC) of continuous glucose monitoring. When the significance of these indices was compared by multiple correlation coefficients in a simple regression analysis, the AUC of continuous glucose monitoring showed the strongest correlation with urinary glucose excretion (multiple correlation coefficient: 0.57). This was followed by the average blood glucose levels by 4 points a day (before meals and before bedtime, 0.48) and the fasting blood glucose levels (0.35). HbA1c showed a relatively weak but significant correlation with urinary glucose excretion (0.29) (Monobe et al. 2021).

Besides blood glucose, other clinical factors are also considered to affect the threshold of glucosuria and the amount of urinary glucose excretion. The renal threshold of glucose excretion is reported to increase in older individuals with diabetes mellitus (Butterfield et al. 1967; Peters and Van Slyke 1946). Similar to previous studies, a significant negative correlation between age and urinary glucose excretion was confirmed by simple regression analysis in individuals with diabetes mellitus (multiple correlation coefficient: −0.24) (Monobe et al. 2021). The relationships between urinary glucose excretion and average blood glucose were studied using

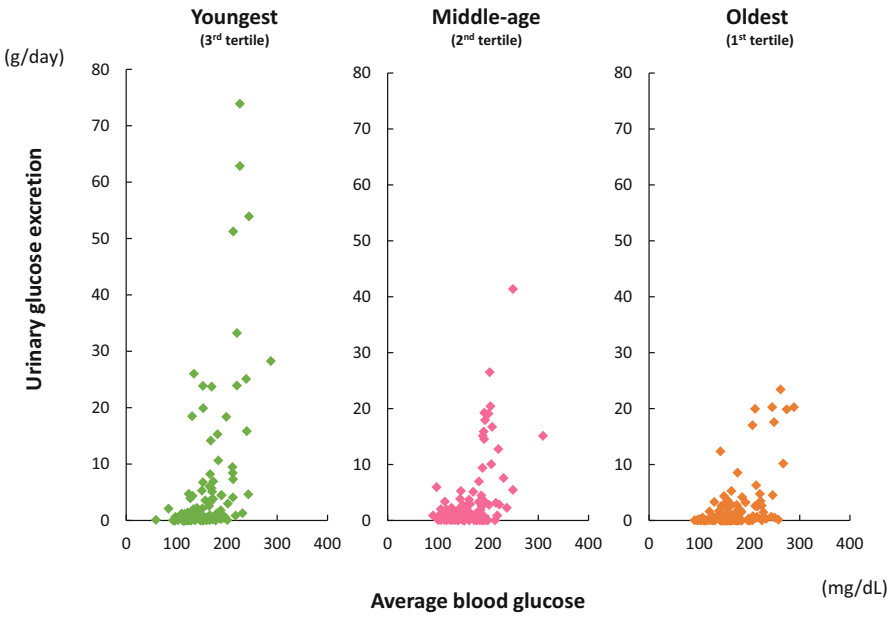

Fig. 1 Correlation between urinary glucose excretion and average blood glucose, stratified according to the age tertiles

age tertiles (Fig. 1). Although urinary glucose excretion seems to decrease with age, inter-individual variations were still observed within each tertile, suggesting the contribution of alternative determinants to the amount of urinary glucose excretion. Since renal function physiologically declines with aging, renal function (estimated glomerular filtration rate [eGFR]) is also an important determinant of urinary glucose excretion. A clear positive correlation was observed between eGFR and urinary glucose excretion (multiple correlation coefficient: 0.31), indicating that the amount of urinary glucose excretion is reduced by a decline in eGFR (Monobe et al. 2021). When the participants in Fig. 1 were stratified according to the tertiles of eGFR, urinary glucose excretion was markedly reduced in the lowest tertile (Fig. 2, right panel). This observation suggests that a decrease in renal function raises the renal threshold of glucose absorption in the proximal tubule due to reduced glomerular filtrate, leading to a decrease in urinary glucose excretion. In contrast, the urinary glucose excretion in preserved renal function, depicted by the highest and middle tertiles of eGFR (Fig. 2, left and middle panels), is likely to be affected by the renal function and the blood glucose levels. These observations were further supported by the evidence that eGFR is the second independent determinant (β, standard partial regression coefficient: 0.28) of urinary glucose excretion by multivariate analysis, following the average blood glucose levels (β 0.41) (Monobe et al. 2021). Contrary to the significant correlation between eGFR and urinary glucose excretion, age was dismissed as an independent variable of urinary glucose excretion, indicating that eGFR is a major determinant of age-dependent reduction of urinary glucose excretion.

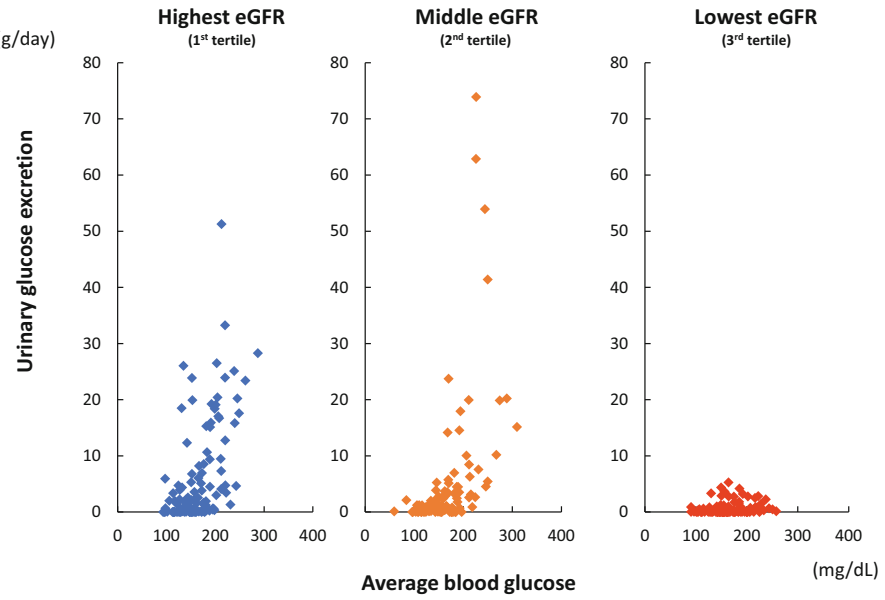

Fig. 2 Correlation between urinary glucose excretion and average blood glucose, stratified according to the eGFR tertiles

Previous studies have consistently reported that the threshold of glucosuria is higher in women than in men (Butterfield et al. 1967; Peters and Van Slyke 1946), suggesting that sex is a critical determinant of urinary glucose excretion. A multivariate analysis has validated that sex was the second strongest determinant (β 0.28) of urinary glucose excretion in patients with diabetes mellitus, following average blood glucose. This indicates that there is a higher urinary glucose excretion in men than in women (Monobe et al. 2021). An animal study reported that the SGLT2 protein was more dominantly expressed in the kidneys of female rats despite the similar expression levels of mRNA in both sexes, highlighting the posttranscriptional regulation by the sex hormones (Sabolic et al. 2012). This observation supports the possible mechanism by which the dominant expression of SGLT2 protein raises the threshold of urinary glucose excretion in females. Monobe et al. also reported that no significant correlation with urinary glucose excretion was observed for the duration of diabetes, urine volume, and body mass index by simple regression analysis (Monobe et al. 2021).

Sodium-Glucose Cotransporters and Glucosuria

Glucose in the glomerular filtrate is reabsorbed at the proximal tubule of the renal cortex by SGLTs. The SGLTs are encoded by the solute carrier 5 (SLC5), a subfamily of sodium/substrate symporter genes (Wright et al. 2007), and transport glucose through the luminal membrane of the epithelial cells in the proximal tubule

by coupling with the inward diffusion of sodium ions. SGLT2, an isoform of SGLT encoded by *SLC5A2*, is almost entirely expressed in the S1 segment of the proximal tubule, which mediates the absorption of more than 90% of the glucose from the glomerular filtrate under physiological conditions. Meanwhile, SGLT1 is expressed in the more distal segments (S2–S3) of the proximal tubule and completely absorbs the residual glucose (less than 10%) from the primary urine (Wright 2001).

The renal threshold of glucose reabsorption is reported to be elevated under diabetic conditions (Freitas et al. 2008; Mogensen 1971; Rahmoune et al. 2005). In the diabetic state, the expression of SGLT2 is upregulated in the renal proximal tubules by three times compared to the healthy controls (Rahmoune et al. 2005). The upregulation of the SGLT2 protein increases the reabsorption of glucose from the glomerular filtrate into circulation, leading to the hyperglycemic state. As a result, abundant glucose flows into the proximal tubule through glomerular filtration under hyperglycemic conditions, and eventually, the high amount of glucose in the primary urine overcomes the upregulated threshold of reabsorption by SGLT proteins.

Mutations in the SGLT2 Gene and Urinary Glucose Excretion

Some genetic mutations in *SLC5A2*, the gene that encodes SGLT2, are responsible for familial renal glucosuria caused by reduced reabsorption of glucose in the proximal tubule, leading to chronic glucosuria even under normal glycemic conditions. Individuals with homozygous or compound heterozygous mutations in *SLC5A2* develop severe glucosuria, while those with heterozygous mutations only present with mild glucosuria (Calado et al. 2006, 2008; Santer et al. 2003). Despite the lifetime presence of prolonged glucosuria, familial renal glucosuria is considered to be a benign condition and is not associated with critical complications or severe outcomes (Santer and Calado 2010). Regarding the mutations of *SLC5A2* in individuals with type 2 diabetes, approximately 3% of the Chinese population have been reported to suffer from renal glucosuria, complicated by type 2 diabetes. Another 3% of individuals showed extremely low urinary glucose excretion with type 2 diabetes, which may affect the diabetes-related phenotypes, such as body mass index and blood glucose levels (Gong et al. 2017). Sequence analysis identified a novel mutation in *SLC5A2*, V359G, in an individual with extremely low urinary glucose excretion. Individuals with lower glucose excretion show higher BMI, prevalence of hypertension, fasting plasma glucose levels, and HOMA-IR (homeostasis model assessment insulin resistance) values than those with renal glucosuria (Gong et al. 2017).

Common Variants of the SGLT2 Gene

In addition to rare mutations, common genetic variants in *SLC5A2* also affect the renal threshold of glucosuria and diabetes-related phenotypes, such as blood glucose levels and even susceptibility to type 2 diabetes (Drexel et al. 2019; Enigk et al. 2011; Monobe et al. 2021; Zimdahl et al. 2017). According to the HapMap database,

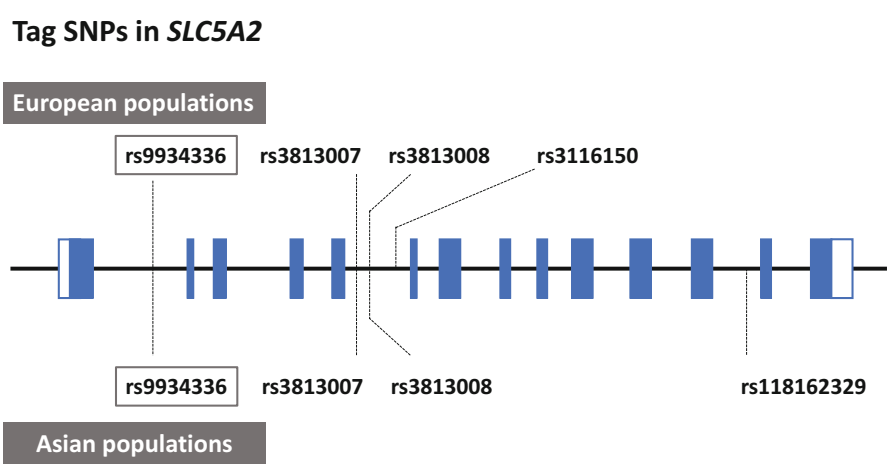

Fig. 3 Tag single nucleotide polymorphisms in *SLC5A2* (SGLT2 gene) between the European and Asian populations. (Reproduced with permission from Noso S: *J Diabetes Investig.* 12: 728–737, 2021)

Enigk et al. have reported that rs9934336, rs3813007, rs3813008, and rs3116150 are the tag single nucleotide polymorphisms (SNPs) of *SLC5A2* in the European populations (Enigk et al. 2011). Among these SNPs, rs9934336, rs3813007, and rs3813008 are the common tag SNPs across ethnicities in European and Asian populations based on the 1000-genome database (Fig. 3) (Enigk et al. 2011; Monobe et al. 2021).

SLC5A2 and Diabetes-Related Phenotypes

With respect to the relationship between the common variants of *SLC5A2* and diabetes-related phenotypes, rs9934336, a tag SNP located in intron 5 of *SLC5A2* (Fig. 3), was reported to be significantly associated with blood glucose levels after glucose load in nondiabetic individuals (normal glucose tolerance, impaired fasting glucose, and impaired glucose tolerance) (Enigk et al. 2011). In this study, a significant reduction was observed in the 30-min and 120-min AUC of blood glucose after the 75-g oral glucose tolerance test in individuals with the A allele of rs9934336. Similarly, Drexel et al. showed that *SLC5A2* rs9934336 was significantly associated with diabetes-related phenotypes in a total cohort study, including non-diabetic individuals and patients with type 2 diabetes, with the A allele of rs9934336. It was associated with lower HbA1c, fasting glucose, and 120-min glucose levels (Drexel et al. 2019). Considering the function of SGLT2 protein encoded by *SLC5A2* in glucose reabsorption at the renal proximal tubule, the *SLC5A2* polymorphisms could be indirectly related to the reduction of the blood glucose level owing to the increased amount of urinary glucose excretion. However, urinary glucose excretion is largely affected by clinical determinants, such as average blood glucose, renal

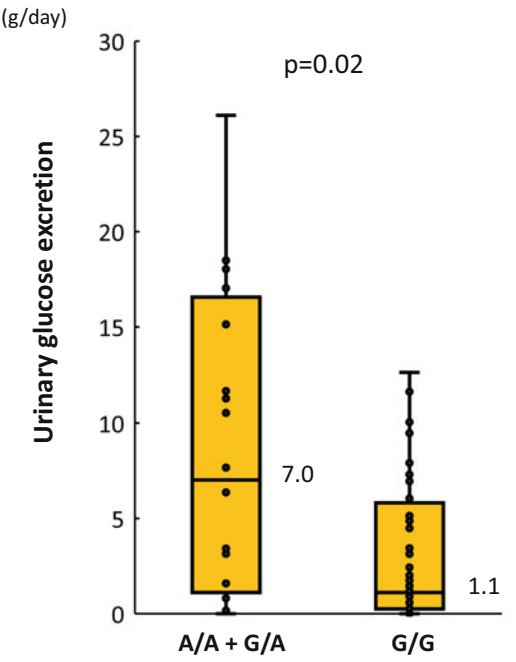

Fig. 4 Urinary glucose excretion in the individuals with the A/A + G/A and G/G genotypes. Mann Whitney-U test. (Reproduced with permission from Noso S: *J Diabetes Investig.* 12: 728–737, 2021)

function, and sex as shown by Monobe et al. (2021). Therefore, these confounding factors should be considered when investigating the contribution of genetic factors. A multiple regression analysis including these clinical determinants clarified that the *SLC5A2* rs9934336 is also an independent determinant ($\beta = 0.17$, $p = 0.02$) of urinary glucose excretion in individuals with diabetes mellitus (Monobe et al. 2021). Monobe et al. reported that the amount of urinary glucose excretion was significantly higher in individuals with A/A + G/A genotypes than in those with the G/G allele (Fig. 4), further supporting the hypothesis that the A allele of rs9934336 contributes to increased urinary glucose excretion and reduced blood glucose levels. With respect to molecular function, rs9934336 was estimated to create a possible splicing enhancer site as an intronic SNP. Alternatively, another functional variant in linkage disequilibrium with rs9934336 may affect SGLT2 protein dysfunction.

Association of SGLT2 Gene with Susceptibility to Type 2 Diabetes Mellitus

In addition to the correlation of a common variant (rs9934336) in *SLC5A2* with blood glucose levels and urinary glucose excretion, several association studies between *SLC5A2* polymorphisms and susceptibility to type 2 diabetes mellitus have been reported to date (Drexel et al. 2019; Enigk et al. 2011; Monobe et al.

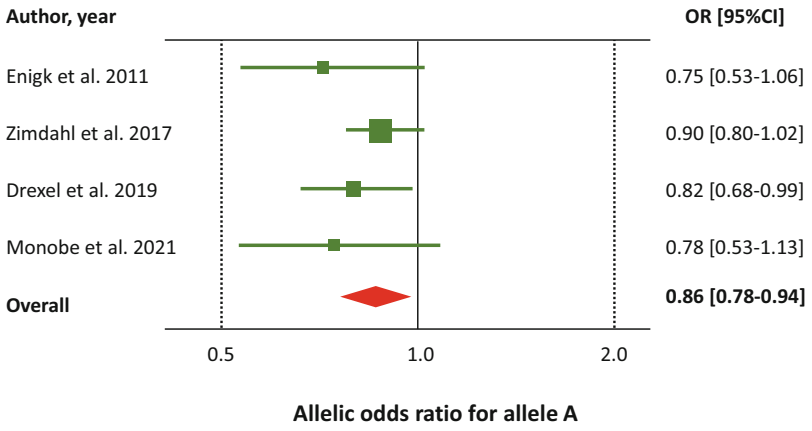

Fig. 5 Meta-analysis of the association studies investigating *SLC5A2* rs9934336 and susceptibility to type 2 diabetes mellitus. (Reproduced with permission from Noso S: *J Diabetes Investig.* 12: 728–737, 2021)

2021; Zimdahl et al. 2017). A meta-analysis of these association studies has clarified that the rs9934336 polymorphism of *SLC5A2* was significantly associated with susceptibility to type 2 diabetes mellitus, highlighting the protective role of the A allele against the development of diabetes mellitus (Fig. 5; summary odds ratio, 0.86; 95% confidence interval, 0.78–0.94, $p<0.002$). Although these studies included the European and Asian populations as distinct ethnicities, no heterogeneity between the studies was observed ($I^2 = 0.0\%$), suggesting that genetic heterogeneity does not exist in the association of *SLC5A2* with susceptibility to type 2 diabetes across different populations. However, there was a difference in the risk allele frequencies (G allele) between the European (74.7%) (Drexel et al. 2019) and Asian populations (87.4%) (Monobe et al. 2021). This indicates that more Asian individuals have reduced urinary glucose excretion. Drexel et al. performed an additional association study of *SLC5A2* variants with the incidence of cardiovascular events, but no significant association was observed for the tag SNPs, including rs9934336. According to a recent meta-analysis of the results from randomized outcome trials, Asian populations may have greater benefit for cardiovascular death and heart failure from SGLT2 inhibitors in comparison with European populations (Lee et al. 2021), suggesting a possible link between higher frequencies of the G allele of rs9934336 and greater benefit of SGLT2 inhibitors in Asian populations. Further studies are needed to clarify the association between the rs9934336 polymorphism and cardiovascular events in Asian populations.

Taken together, the data regarding *SLC5A2* rs9934336 polymorphism with diabetes-related phenotypes indicate that the A allele of rs9934336 is likely to be associated with lower blood glucose levels due to the increased urinary glucose excretion caused by SGLT2 protein dysfunction. As such, it acts as a protective allele against the development of type 2 diabetes mellitus (Fig. 6).

Fig. 6 Schema of links of alleles of SLC5A2 rs9934336 and diabetes-related phenotypes

Application to Prediction

SLC5A2 rs9934336 Polymorphism as a Genetic Marker of Diabetes-Related Phenotypes

As described above, accumulating evidence strongly indicates that the *SLC5A2* rs9934336 polymorphism could be a useful biomarker in predicting the amount of urinary glucose excretion, blood glucose levels, and susceptibility to type 2 diabetes. Based on these hypotheses, individuals harboring the G allele of rs9934336 could be predicted to have reduced urinary glucose excretion, leading to hyperglycemia and type 2 diabetes mellitus in the future. Hwang et al. reported that a better response to SGLT2 inhibitors is expected for individuals with reduced urinary glucose excretion in the Asian population (Hwang et al. 2019). Therefore, this novel biomarker may contribute to the selection of diabetic individuals with better response to pharmacologic intervention by SGLT2 inhibitors and the establishment of prediction and prevention of cardiovascular events and heart failure as chronic complications of diabetes mellitus.

Mini-Dictionary of Terms

- **Glucosuria**: Glucose in the urine resulting from excess glucose beyond the threshold of glucose reabsorption at the renal proximal tubule.
- **Gluconeogenesis**: A metabolic pathway that generates glucose from non-carbohydrate carbon substrates. It mainly occurs in the liver and kidney.
- **Glomerular filtrate**: The blood is filtered across the capillary walls of the glomerulus in the kidney into Bowman's capsule. The filtrate then enters the renal proximal tubule of the nephron.

- **Primary urine**: The fraction of blood plasma that is filtered by the glomerulus, also known as the glomerular filtrate.
- **Sodium-glucose transport protein 2 (SGLT2)**: Encoded by solute carrier 5 (SLC5), this transports glucose through the luminal membrane of the epithelial cells in the proximal tubule by coupling with the inward diffusion of sodium ions. SGLT2, an isoform of SGLT that is encoded by *SLC5A2*, is almost entirely expressed in the S1 segment of the proximal tubule.
- **Single nucleotide polymorphism**: A single nucleotide variant in the genome sequence, with a minor allele frequency of more than 1% in the general population.

Key Facts of Urinary Glucose Excretion

- Urinary glucose excretion is determined by the average blood glucose, eGFR, sex, and *SLC5A2* rs9934336.
- *SLC5A2* rs9934336 is associated with blood glucose levels, urinary glucose excretion, and susceptibility to type 2 diabetes mellitus.

Summary Points

- Urinary glucose excretion is determined by average blood glucose, renal function, sex, and *SLC5A2* rs9934336.
- *SLC4A2* rs9934336 is associated with blood glucose, urinary glucose excretion, and the susceptibility to type 2 diabetes mellitus.
- Lower urinary glucose excretion, predicted by *SLC5A2* rs9934336, is a possible biomarker for the development of type 2 diabetes mellitus and the efficacy of SGLT2 inhibitor.

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Secreted Frizzled-Related Proteins 4 and 5: What They Are and Can They Be Used as a Biomarker in Gestational Diabetes Mellitus

30

Rik H. J. Beernink and Joost H. N. Schuitemaker

Contents

Glucose Homeostasis During Pregnancy 631
Gestational Diabetes Mellitus 631
Adverse Pregnancy Outcomes GDM and Treatment 632
Secreted Frizzled-Related Protein Family 633
Secreted Frizzled-Related Protein Family During Pregnancy 635
sFRP4 and sFRP5 in Glucose Homeostasis 636
Biomarker for Gestational Diabetes Mellitus 640
Applications to Prognosis 641
Applications to Other Diseases or Conditions 642
Mini-Dictionary of Terms 642
Key Facts of Secreted Frizzled-Related Proteins 4 and 5: What They Are and Can They Be Used as a Biomarker in Gestational Diabetes Mellitus 643
Key Facts of Gestational Diabetes Mellitus 643
Key Facts of Secreted Frizzled-Related Proteins (sFRPs) 643
Summary Points 643
References 644

Competing Interests
Both authors are employed by IQ Products BV.

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Abstract

During pregnancy the maternal metabolic profile changes from an anabolic to a catabolic state, however some women are unable to sufficiently adapt their metabolic profile and develop a *de novo* hyperglycemic state, called gestational diabetes mellitus (GDM). Since the risk of adverse pregnancy outcomes related to GDM are already present during the diagnosis of GDM, there is a search for a method to earlier predict its development in order to start a (therapeutic) intervention. Secreted frizzled-related protein 4 (sFRP4) and secreted frizzled-related protein 5 (sFRP5) are two antagonistic proteins for the Wingless and Int-1 (Wnt) signaling pathway and both are possibly involved in the development of diabetes. In addition, they are associated with the development of the placenta and differential expression of both proteins is observed in various pregnancy-related disorders. Therefore, this chapter describes the glucose homeostasis during pregnancy, the definition of GDM, and evaluate the role of sFRP4 and sFRP5 in the development of diabetes and their (potential) association with the development of GDM.

Keywords

Secreted frizzled-related protein 4 · Secreted frizzled-related protein 5 · Wingless and Int-1 signaling pathways · Glucose homeostasis during pregnancy · Gestational diabetes mellitus

Abbreviations

APC	Adenomatosis polyposis coli
BMI	Body mass index
CAMKII	Calmodulin-dependent kinase II
CK-1α	Casein kinase-1
Dsh	Dishevelled
FZD	Frizzled transmembrane receptors
GDM	Gestational diabetes mellitus
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter 4
GSK3β	Glycogen synthase kinase-3 beta
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IL-1β	Interleukin-1β
IRS-1	Insulin receptor substrate-1
JNK	C-Jun N-terminal kinase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OGTT	Oral glucose tolerance test
PCP pathway	Planar cell polarity pathway
PI3k	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
sFRP4	Secreted frizzled-related protein 4
sFRP5	Secreted frizzled-related protein 5
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCF	T cell factor
TNFα	Tumor necrosis factor α
Wnt pathway	Wingless and Int-1-type signaling pathways
Wnt5a	Noncanonical Wingless-type family member 5a

Glucose Homeostasis During Pregnancy

In general, a glucose tolerance can be caused through two ways: a reduced tissue sensitivity for insulin or insufficient secretion of insulin. During pregnancy both physiological actions are changing in order to fulfill the metabolic demands of the mother and offspring. The maternal metabolic profile during pregnancy can be roughly divided into an anabolic and catabolic state, whereby the early gestation has a more anabolic characteristic and the late gestation a more catabolic characteristic (McIntyre et al. 2019). The early anabolic state results in more maternal fat storage in order to fulfill the fetal and maternal nutrition demands at the end of pregnancy. Throughout the pregnancy, maternal tissue sensitivity for insulin gradually decreases which might even go to 50% of normal expected values (Sonagra et al. 2014; Catalano et al. 1998). This decline is partly mediated by hormonal factors of the placenta, such as increased levels of estrogen, progesterone, and lactogen. To (partly) compensate this reduced sensitivity, there is an increased production of insulin to mediate a normoglycemic environment, but overall there is still an insulin-resistant state at the end of the pregnancy (Catalano et al. 1999). This resistance will result in more maternal use of fats for her energy demands and more available carbohydrates for the rapidly growing fetus (Sonagra et al. 2014). However, some women develop an abnormal insulin sensitivity, are unable to compensate the decreasing sensitivity with sufficient insulin secretion or experience a combination of these two, and, subsequently, develop a hyperglycemic state during pregnancy, called gestational diabetes mellitus (GDM) (McIntyre et al. 2019).

Gestational Diabetes Mellitus

GDM is the detection of a *de novo* hyperglycemia during pregnancy, generally detected in the late second trimester or early third trimester and the disorder resolves most of the time after birth (McIntyre et al. 2019). Whereas type 1 diabetes mellitus (T1DM) is an autoimmune disease destroying the β-cells and type 2 diabetes mellitus (T2DM) is characterized by an inability to produce sufficient insulin to

compensate the present insulin resistance, GDM is most likely caused by a combination of β-cell dysfunction, insulin resistance, placental-hormones, oxidative stress, and/or general present inflammatory state during pregnancy (Plows et al. 2018; Chiefari et al. 2017). For example, minor β-cell defects are normally not directly exposed in nonpregnant women, but they can be revealed during a metabolic stress condition, such as pregnancy. Next, the placenta is a major regulator of glucose homeostasis during pregnancy through the secretion of hormones and cytokines. Recent studies also identified that GDM placentas and uncomplicated placentas are significantly different on protein levels (Plows et al. 2018). Removal of this metabolic stress and/or the placenta is most likely the reason why GDM resolves postpregnancy and therefore studies suggest that GDM might be different from T1DM and T2DM (Plows et al. 2018; Egan et al. 2020; Chiefari et al. 2017).

Currently, there is no worldwide agreement of an uniform diagnostic definition for GDM (Benhalima et al. 2015). For example, GDM is diagnosed in the Netherlands when there is a fasting glucose level of $\geq$7.0 mmol/L or after 2 h a glucose level of $\geq$7.8 mmol/L after taking a 75 g oral glucose tolerance test (OGTT). Whereas other countries use other criteria, such as the International Association of Diabetes and Pregnancy Study Groups (IADPSG), where GDM is diagnosed when a fasting glucose of $\geq$5.1 mmol/l or after an OGTT, glucose levels of $\geq$10.0 mmol/l after 1 h or $\geq$8.5 mmol/l after 2 h are present. According to most guidelines, an OGTT is currently only performed based on maternal risk factors such as advanced maternal age, body mass index (BMI), ethnicity, or first-degree family history of diabetes mellitus (McIntyre et al. 2019). Due to this nonuniformity and the diverged presence of risk factors around the world, national GDM prevalence rates are ranging from 1 to >30% (McIntyre et al. 2019).

Adverse Pregnancy Outcomes GDM and Treatment

Diabetes during pregnancy increases the risk of adverse pregnancy outcomes for both mother and offspring with, for example, increased risk for preeclampsia and macrosomia (i.e., excessive fetal growth). This macrosomia is caused by the hyperglycemic environment during GDM, which is the main nutrient source for the fetus, and free fatty acids (FFAs) from maternal lipoproteins. These maternal FFAs cross the placenta and together with the fetal metabolized FFAs from the hyperglycemic environment they are stored as lipids in the fetal white adipose tissue with macrosomia as a result (McIntyre et al. 2019). More important, this macrosomia is often already observed when GDM is diagnosed with an OGTT (Sovio et al. 2016). Therefore, there is a search for a method to earlier predict the development of GDM, since this might give the possibility for more intensive pregnancy monitoring with extra health care visits, glucose monitoring, and fetal ultrasonography. In 2013, the World Health Organization showed in a systematic review that treatment of GDM reduces the direct adverse pregnancy outcomes (Guideline WHO 2014). Management of GDM starts with general lifestyle advices such as a healthy diet and regular exercise and if unsuccessful, the women are set on glucose-lowering

medication (Rasmussen et al. 2020). Moreover, GDM women and their offspring have a higher risk of cardiovascular diseases or metabolic syndrome later in life (Farahvar et al. 2019). Thus, diagnosis gives the possibility to (better) monitor these women and offspring more intensively than uncomplicated pregnancies after birth.

Multiple studies have already shown an improved early risk assessment of GDM by including one or more biochemical markers in the current prediction models, such as single-nucleotide polymorphism of genes, metabolites, or proteins, likely because they are more related to the pathophysiological pathways of GDM than just demographic risk factors (Abell et al. 2015; White et al. 2016; McIntyre et al. 2019). One intensively researched protein group are adipokines, which are proteins secreted by adipocytes in white adipose tissue and commonly associated with metabolic diseases, however no adipokine did not yet showed sufficient clinical utility for early GDM prediction (Al-Badri et al. 2015). Recently published studies suggest that secreted frizzled receptor 4 (sFRP4) and secreted frizzled receptor 5 (sFRP5) might also contribute to the prediction of GDM in the first and early second trimester (Oztas et al. 2016; Schuitemaker et al. 2020; Baldane et al. 2018). Although they are described as an adipokine, both are also expressed by other organs such as the pancreas and endometrium (Pawar and Rao 2018; Wang et al. 2020).

Secreted Frizzled-Related Protein Family

In total, the secreted frizzled-related protein (sFRP) family comprises five secreted glycoproteins, sFRP1 to sFRP5, and all are modulators of the Wingless and Int-1-type (Wnt) signaling pathway. The protein family consists of two subgroups on the basis of sequence homology; sFRP1, sFRP2, and sFRP5 from the subfamily 1Sarp and sFRP3 and sFRP4 from the subfamily FrzB (Pawar and Rao 2018). Wnts agonists are ligands which can bind to frizzled transmembrane receptors (FZD) and sFRPs interact either with Wnts ligand to prevent them from binding to the FZD or they block the receptor by binding the FZD (Surana et al. 2014). The Wnt pathway can be subdivided in three biological pathways; the β-catenin-dependent (canonical) pathway and two β-catenin-independent (noncanonical) pathways and in all three pathways the cytosolic protein Dishevelled (Dvl) plays a crucial role. Dvl is a cytoplasmic phosphoprotein which is activated after FZD activation and regulates the downstream effect in the cell (Gao and Chen 2010). All three pathways are not autonomous and there are degrees of overlap and interaction (Weidinger and Moon 2003).

In the inactivated state of the canonical pathway (i.e., β-catenin-dependent pathway), β-catenin is bound by a large protein complex (Fig. 1a). This multiprotein complex consists of serine threonine kinase glycogen synthase kinase-3 (GSK-3), axin, adenomatosis polyposis coli (APC), casein kinase-1 (CK-1α), and phosphorylated ERK (pERK). In this protein complex β-catenin gets phosphorylated, ubiquitinated, and subsequently degraded by a proteasome. When the Wnt agonist binds the extracellular N-terminal cysteine-rich domain of a FZD and co-receptor LRP5/6, the canonical pathway is activated. Upon activation of the receptors, their signal is transmitted via a direct interaction to the intracellular Dvl protein and this

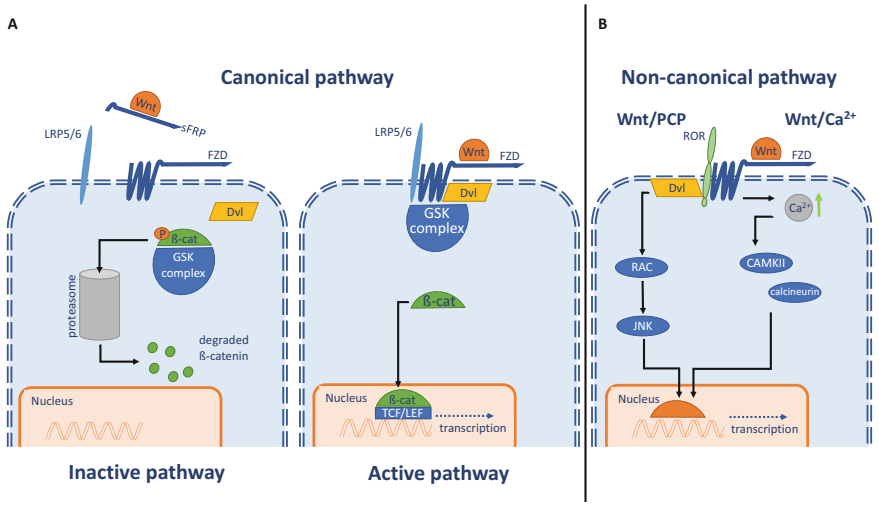

Fig. 1 Signaling pathway of nonactivated canonical (**a**), activated canonical (**a**), and noncanonical (**b**) Wnt signaling pathways. In the absence of Wnt or in the event of antagonism by sFRP (**a**), the cytosolic concentration of free β-cat is controlled by the GSK complex in the canonical Wnt signaling pathway. This GSK complex consists of GSK-3, axin, APC, CK-1α, and pERK. In this protein complex β-cat gets phosphorylated, ubiquitinated, and subsequently degraded by a proteosome. When a Wnt agonist binds the extracellular N-terminal cysteine-rich domain of a FZD and co-receptor LRP5/6, the canonical pathway is activated. Upon activation of the receptors, their signal is transmitted via a direct interaction to the cytosolic Dvl protein and this disrupts the GSK destruction complex, preventing the degradation of β-cat. Without degradation, the β-cat is translocated to the nucleus and interacts with a member of the TCF family to form a complex. The formation of this complex will result in gene transcription. The noncanonical pathway consists of the Wnt/PCP and Wnt/Ca^{2+}pathway and in both signaling pathways β-cat is not involved (**b**). Upon activation of Wnt/PCP pathway with the co-receptor ROR2, the receptor recruits the cytosolic Dvl protein. Dvl can subsequently form a complex with RAC and this activates JNK. In the Wnt/Ca^{2+} pathway, activation will again result in activated Dvl followed by an intracellular Ca^{2+} release by the endoplasmic reticulum after several kinase steps. These increased Ca^{2+} levels activate, among other factors, calcineurin and CAMKII and subsequently the activation of the transcription factor NFκB next to other transcription factors

disrupts the GSK destruction complex. By doing this, the GSK complex is unable to phosphorylate β-catenin and without degradation, the β-catenin is translocated to the nucleus. In the nucleus the protein interacts with a member of the T cell factor (TCF) family to form a complex. The formation of this complex will result in promotion of Wnt-responsive genes such as c-Myc, cyclin D1, and PPARδ, which are responsible for, among other biological processes, increased cell division, regulation of cell cycle progression, and energy metabolism. The TCF family consists of four members and in absence of β-catenin there is no transcription of the genes (Jin 2008).

The noncanonical pathways operate completely independent of β-catenin and consist of the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway, whereby activation of the latter results in activated phospholipase C (PLC) and calcium release from the endoplasmic reticulum. This release can activate, among

other factors, calmodulin-dependent kinase II (CAMKII) and calcineurin, which will induce the transcription of genes (Fig. 1b). The PCP pathway is activated via binding of Wnt to the FZD receptor and forming a complex with the ROR co-receptor (Fig. 1b). Through several kinase steps and complexes, the cytoskeleton and cell migration can be regulated, but activation can also result in activation of C-Jun N-terminal kinase (JNK). JNK is a stress-activated kinase with increased activity in metabolic disorders (Bretón-Romero et al. 2016). All three Wnt pathways play an important role in regulating cell proliferation, fetal development, maintenance, and differentiation of stem/progenitor cells, but also dysregulation of the Wnt signaling has been associated with several diseases such as carcinoma, myocardial infarction, diabetes, and pregnancy-related disorders (Luo et al. 2007; Clevers and Nusse 2012; Tepekoy et al. 2015). Although the Wnt pathway is physiologically widespread, the focus in this chapter will be on their role during pregnancy and the development of (gestational) diabetes mellitus.

Secreted Frizzled-Related Protein Family During Pregnancy

Studies suggest that the sFRP family play a crucial role during pregnancy, especially in the preimplantation, implantation of the fetus, and development of the placenta (Tepekoy et al. 2015). Studies also demonstrated that 14 of the 19 known Wnt ligands and 8 frizzled receptors are present in the human placenta, which supports this idea, and another study also concluded that dysregulation of sFRPs are associated with infertility, endometriosis, and choriocarcinoma (Sonderegger et al. 2007, 2010).

Of all five identified secreted glycoproteins members, sFRP4 and sFRP5 are not only related to the implantation and development of the placenta, but also their differential expression is associated with pregnancy-related disorders. The sFRP4 protein consists of 346 amino acids and contains a cysteine-rich domain with homologous properties to the extracellular domain of the frizzled proteins, making it an antagonist for the Wnt signaling pathway. The protein is also known by its synonym "frizzled-related protein human endometrium." Further, its expression by the pancreas is stimulated by interleukin-1β (IL-1β) and it is suggested that the protein is regulated by estrogen and progesterone, both important proteins during the menstrual cycle and pregnancy (Mahdi et al. 2012; Fujita et al. 2002). Increased levels of SFRP4 were, among other disorders, detected in reduced placental growth and preeclampsia, which is a pregnancy-related disorder wherein the penetration of the trophoblast cells into the maternal myometrium is insufficient (White et al. 2009; Hewitt et al. 2006). Trophoblast cells are the first cells to differentiate from the fertilized egg and develop into a large part of the placenta after implementation. An insufficient penetration will result in an abnormal placental development and later during pregnancy in oxidative stress and an inflammatory state (Rana et al. 2019). sFRP5 contains 311 amino acids and also has a Wnt antagonistic cysteine-rich domain. The protein is also known as secreted apoptosis-related protein 3 and sFRP5 knockout mice also showed insufficient trophoblast differentiation with

oxidative stress and inflammation as result (Bao et al. 2020). Both oxidative stress and persistent low-grade inflammation are also observed in GDM pregnancies (Lappas et al. 2011; Hoch et al. 2019).

sFRP4 and sFRP5 in Glucose Homeostasis

Next to a possible biological role of sFRP4 and sFRP5 in oxidative stress and inflammation during pregnancy, the Wnt signaling pathway is also associated with diabetes. Its pathways are involved in pancreatic β-cell proliferation and insulin production, and also in the production of glucagon-like peptide-1 (GLP-1), which is an intestinal hormone and responsible for rapid insulin secretion after a meal prior to the elevation of blood glucose levels (Jin 2008; Chiang et al. 2012). Recent genome studies also showed that polymorphisms in TCF-4 is associated with a major risk of T2DM and reduced insulin secretion (Grant et al. 2006; Schäfer et al. 2007). In addition, studies specifically associated differential expression of sFRP4 and sFRP5 with the development of diabetes (Liu et al. 2018; Ouchi et al. 2010; Wang et al. 2020; Pawar and Rao 2018; Wilson 2013). For sFRP4, increased levels are widely published to be associated with an increased risk of diabetes (Bergmann and Sypniewska 2014; Pawar and Rao 2018; Wilson 2013; Bukhari et al. 2019). sFRP4 has been described to a lesser extent as adipokine as sFRP5 and it plays a role in the glucose metabolism through two biological pathways. First, the protein suppresses the exocytosis of insulin through reducing the expression of calcium ion channels in pancreatic β-cells (Mahdi et al. 2012; Bergmann and Sypniewska 2014) (Fig. 2a). Normally, the release of insulin is triggered by the metabolism of glucose, which increases the ADP/ATP ratio within the cell. This increase closes the ATP-sensitive potassium channels and, as result, the potential across the membrane becomes more positive through the intracellular accumulation of the potassium ions. Because of this potential change, voltage-dependent calcium channels open and cause a calcium influx. This influx subsequently triggers insulin vesicle exocytosis. It is not completely clear through which signaling pathway this reduction of calcium ion channels is realized, but increased sFRP4 results in decreased cytosolic active β-catenin and studies showed that β-catenin is also involved in surface expression of calcium-activated potassium channels (Bian et al. 2011). Further, Sorrenson et al. (2016) showed in *in vitro* β-cell models and in *in vivo* isolated mice β-cells and pancreatic islets that reduced β-catenin protein levels suppress glucose- and incretin-stimulated insulin secretion by dysregulating the localization and fusion of insulin vesicles with the plasma membrane (Fig. 2a). This regulation was independent of the transcriptional effect of the β-catenin/TCF complex and the authors suggest that reduced β-catenin might be responsible for attenuated intracellular actin remodeling, which is required for insulin secretion. Thus, antagonizing the Wnt/β-catenin by increased sFRP4 levels in diabetes might result in less active cytosolic β-catenin and dysregulated insulin translocation and secretion.

The uptake of glucose, and therefore the sensitivity for insulin, is regulated by insulin target tissue. Normally, insulin activates the transmembrane tyrosine kinase

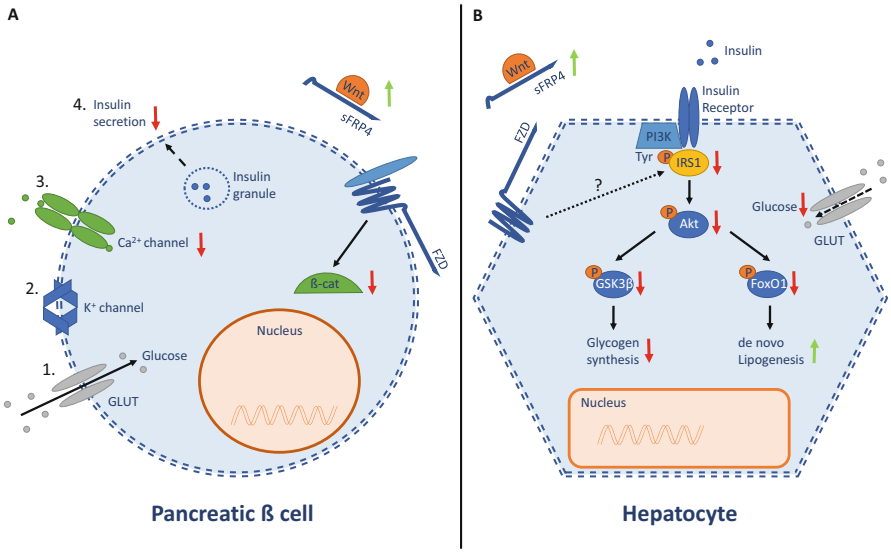

Fig. 2 The effect of increased sFRP4 levels on the glucose homeostasis in pancreatic ß-cells (**a**) and hepatocytes (**b**). Increased sFRP4 affects insulin secretion in pancreatic ß-cells in two ways (**a**). Normally, the release of insulin is triggered by the metabolism of glucose, which increases the ADP/ATP ratio within the cell (1), and this increase closes the ATP-sensitive potassium channels (2). As result, the potential across the membrane becomes more positive through the intracellular accumulation of the potassium ions. Because of this potential change, voltage-dependent Ca^{2+} channels open and cause a Ca^{2+} influx (3). This influx subsequently triggers insulin vesicle exocytosis (4). Although the exact biological pathway is unknown, increased sFRP4 levels are associated with reduced Ca^{2+} channel expression in pancreatic ß-cells and thereby a reduced capability to secrete insulin. Second, increased sFRP4 will antagonize the Wnt/beta-catenin pathway resulting in reduced cytosolic active beta-catenin and beta-catenin is needed for the translocation of insulin vesicles to the cell membrane. In hepatocytes, increased sFRP4 levels affect the glucose homeostasis and lipogenesis (**b**). The responsible receptor and mediators for these effects are not yet known, but sFRP4-stimulated hepatocytes show decreased levels of IRS-1. Normally, IRS-1 activation will eventually result in glucose uptake after various kinase reactions. In addition, IRS-1 also activates Akt and this kinase is responsible for the deactivation of GSK3β through serine phosphorylation and activation of FoxO1. Reduced deactivation of GSK3β and reduced activation of FoxO1 are associated with decreased glycogen synthesis and increased lipogenesis, respectively

insulin receptor on target tissue through autophosphorylation of its intracellular tyrosine residues (Lee and Pilch 1994). This generates an intracellular binding site for insulin receptor substrate 1 (IRS-1), which is also subsequently activated through tyrosine phosphorylation. Tyrosine phosphorylated IRS-1 will interact and form a complex with phosphoinositide 3-kinase (PI3k) and this kinase will converse phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by adding another phosphate group. The PIP_3 will activate several kinases, including Akt (i.e., protein kinase B), which will translocate glucose transporter (GLUT) vesicles to the cell membrane. This translocation will eventually result in diffusion of glucose into the cell. Two other proteins that are phosphorylated

by Akt are forkhead box protein O1 (FoxO1) and glycogen synthase kinase-3 beta (GSK3β). FoxO1 contributes to multiple metabolic pathways to regulate systemic and hepatic glucose metabolism and GSK3β plays a role in glycogen synthesis (Zhang et al. 2006; Beurel et al. 2015). To note, GSK3β also has an important role in the phosphorylation of β-catenin and the subsequent degradation. Next to the antagonizing effect of insulin on glucose production by the liver, insulin also promotes fatty acid synthesis (i.e., lipogenesis) in the liver.

Hörbelt et al. (2019) showed in *in vitro* and *in vivo* models that sFRP4 is associated with both increased insulin resistance and lipogenesis through reduced expression of (activated) IRS-1, GSK3β, and FoxO1 in hepatocytes (i.e., liver cells) (Fig. 2b). Reduced expression of IRS-1 will eventually result in reduced Akt activation, but also in decreased GLUT translocation and reduced glucose influx. Reduced Akt levels will subsequently result in reduced phosphorylated GSK3β and FoxO1. While most kinases are activated after phosphorylation, both GSK3α and GSK3β are inactivated after serine phosphorylation (Lee and Kim 2007). Less and inactivation of GSK3β is subsequently responsible for reduced enzymatic conversion of glucose to glycogen, which might keep blood glucose levels high. Next to FoxO1's effect on hepatic glucose production, it is demonstrated that it has a role in lipid synthesis since inhibition of FoxO1 in isolated hepatocytes was needed for increased lipogenesis (Matsumoto et al. 2006; Titchenell et al. 2016). The observed reduction of FoxO1 by sFRP4 in the study of Hörbelt et al. (2019) might be an alternative explanation for the insulin paradox which is observed in diabetes; insulin fails to suppress glucose levels through the liver, since the liver is considered insulin resistant, but on the other hand the other biological effect of insulin, lipogenesis, is increased in a diabetic liver (Titchenell et al. 2016). This increased lipogenesis might be caused by a pathway which does not depend on insulin, such as increased sFRP4 levels. However, it was not investigated in this study via which receptor and mediators sFRP4 caused these effects. Moreover, increased sFRP4 levels are already observed several years before the diagnosis of T2DM and this is the reason why sFRP4 is a possible marker of early pancreatic β-cell dysfunction, insulin resistance, and/or the development of diabetes (Mahdi et al. 2012).

In diabetes and obesity, decreased levels of sFRP5 are in majority present, though also contradictory results are available (Oztas et al. 2016; Wang et al. 2020). Compared to sFRP4, sFRP5 is a more typical adipokine and although the effect of sFRP5 in the Wnt signaling pathway is widespread, its association with obesity, inflammation, and metabolic complications in literature is mainly related to its interaction with Wingless-type family member 5a (Wnt5a) in both noncanonical signaling pathways (Ouchi et al. 2010; Koutaki et al. 2021). The activation of the noncanonical Wnt5A/PCP will result in activated JNK and when it is activated, it can phosphorylate various serine amino acids of IRS-1, such as serine 307 and serine 312 (Lee et al. 2003; Aye et al. 2013). This phosphorylation blocks the possibility to activate IRS-1 through tyrosine phosphorylation by the insulin receptor (Fig. 3). After insulin binds and activates the insulin receptor, tyrosine phosphorylation of IRS-1 is needed to recruit PI3k and to eventually take up glucose and produce glycogen after various intrinsic tyrosine kinase activities (Liu et al. 2018; Draznin

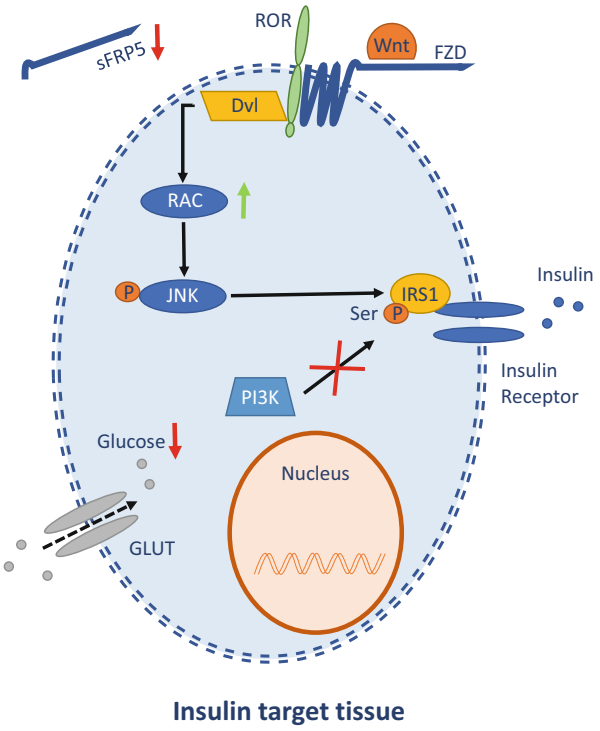

Fig. 3 The effect of decreased sFRP5 levels on the glucose homeostasis in insulin target tissue. Decreased sFRP5 levels are in insulin target tissue responsible for more Wnt/PCP activation. This results in activated JNK and serine phosphorylation of IRS-1. By doing this, the IRS-1 is blocked and cannot interact anymore with PI3K. At the end, this will result in less insulin-sensitive tissue and reduced glucose uptake

2006). Thus, the observed decreased sFRP5 levels in diabetes and obesity cause less antagonism of Wnt5a and, therefore, more activated JNK is present, which will result in more blocked IRS-1 (Wang et al. 2020; Liu et al. 2018). This effect of decreased sFRP5 levels can be found in all insulin target tissues: liver, skeletal muscle, and adipose cells. Blocking of IRS-1 through serine phosphorylation is also caused by pro-inflammatory factors, such as tumor necrosis factor α (TNFα) and IL-1β. Both factors are also increased during diabetes and associated with the development of insulin resistance (Akash et al. 2018; Jager et al. 2007). In addition, IL-1β is negatively correlated with sFRP5 levels and it strongly induces sFRP4 expression in the pancreatic β-cells (Liu et al. 2018; Plows et al. 2018; Mahdi et al. 2012). Next to decreased IRS-1 expression, decreased IRS-1 tyrosine phosphorylation is also observed in skeletal muscle tissue of GDM pregnancies, possibly caused by increased sFRP4 and decreased sFRP5 levels, respectively, next to other factors (Friedman et al. 1999). Moreover, there was a significant lower glucose uptake of 25% in these tissues.

Besides its effect on insulin resistance, sFRP5 also has an anti-inflammatory effect and therefore, decreased sFRP5 levels are associated with a pro-inflammatory state (Koutaki et al. 2021). Fuster et al. (2015) concluded that the noncanonical pathway through Wnt5A contributes to obesity-induced insulin

resistance independent of adipogenesis and adipose tissue expansion. Through less antagonizing of Wnt5A, the Wnt5A/Ca^{2+} and Wnt5A/PCP are more activated and this results in increased transcription of pro-inflammatory genes such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) with insulin resistance in adipose tissue as a result (De 2011; Fuster et al. 2015). Ouchi et al. (2010) also confirmed this anti-inflammatory effect in sFRP5 knockout mice by feeding them a high-fat diet, which resulted in increased adipose tissue inflammation, metabolic dysfunction, and also insulin resistance. After injection of sFRP5, tissue inflammation reduced and insulin sensitivity improved. This dual biological effect of sFRP5 on the glucose homeostasis and regulation of inflammatory factors can also be linked to the complex interplay between insulin resistance and a chronic low-grade inflammation which is observed in diabetes (Wang et al. 2020). All these published *in vitro*, *in vivo*, and human cohort studies indicate that both sFRP4 and sFRP5 might have a role in the development of diabetes. Together with the fact that sFRP4 and sFRP5 are possibly involved in a successful pregnancy, both proteins might also be associated with the development of GDM.

Biomarker for Gestational Diabetes Mellitus

sFRP4 is in three studies described in relation with GDM. Baldane et al. (2018) showed in a cross-sectional case-control cohort study with 41 control pregnancies and 35 GDM pregnant women an increased level of sFRP4 in the late second trimester in overweight women (BMI 29.07 ± 3.26 and 28.33 ± 4.01 kg/m^2, GDM vs controls, respectively). Samples were collected at the moment of OGTT, so in these cases GDM was already present. Next to increased sFRP4 levels, increased prorenin levels were also observed. Yuan et al. (2018) performed a longitudinal study with in total 526 pregnant women, whereof 97 cases were diagnosed with GDM. Longitudinal blood samples were collected at 16–18 weeks, 24–28 weeks, and just before/during delivery (i.e., 37–41 weeks). sFRP4 levels were significantly increased in GDM pregnancies compared to the control group at all time points. In both groups, the levels were significantly lower during delivery (i.e., 37–41 weeks) compared to 16–18 weeks and 24–28 weeks collected samples. This decrease might be related to the fact that the pregnant women in both groups went from an anabolic to catabolic state in this period to better fulfill the nutritional demand of the offspring. Since BMI is one of the major predictors to develop GDM and this study was performed with a lean BMI cohort (22.00 ± 3.43 and 20.98 ± 2.80 kg/m^2 for GDM and control pregnancies, respectively), it is interesting that sFRP4 levels still showed a distinction between GDM and uncomplicated pregnancies in this cohort. To note, both discussed studies were performed in the second trimester or later and a marker should, preferably, have a predictive value in the first trimester, since this gives the possibility to better monitor high-risk pregnancies and the glucose levels of these pregnancies. Second, the risk of adverse pregnancy outcomes of GDM are, in most cases, already present at the moment of GDM diagnosis in the second trimester or later (Sovio et al. 2016). Schuitemaker

et al. (2020) performed a nested case-control study in the first trimester, whereby 50 GDM pregnancies and 100 control pregnancies were included. To note, the pregnancies were matched on maternal age (± 2 years) and BMI (± 2 kg/m^2), which are both major risk factors for the development of GDM. In this slightly overweight cohort (26.6 ± 5.6 and 26.2 ± 5.0 kg/m^2), again significantly increased sFRP4 levels were observed but this time in the first trimester.

For sFRP5, there is only one study available which evaluated whether sFRP5 levels are significantly different in GDM pregnancies compared to uncomplicated pregnancies (Oztas et al. 2016). This study was a prospective, cross-sectional, case-control study with 40 GDM pregnancies and 44 control pregnancies. Analyzed samples were collected in the first trimester (i.e., 10–14 weeks) and women were matched on age and prepregnancy BMI. In this study there was an association of decreased sFRP5 levels with the development of GDM later during pregnancy, but no association was observed to predict adverse pregnancy outcomes related to GDM. The decreased levels of sFRP5 in GDM matched with another well-investigated anti-inflammatory biomarker for GDM in the first trimester, adiponectin (Plows et al. 2018; Lorenzo-Almorós et al. 2019). However, caution should be taken when drawing conclusions, as there is only one publication available about sFRP5 and GDM.

In conclusion, a handful studies currently suggest a predictive role of sFRP4 and sFRP5 in the first trimester for the development of GDM. It is important to note that the clinical presentation of GDM is still absent in the first trimester and a timely (therapeutic) intervention is beneficial for the adverse pregnancy outcomes. Since both proteins are already well investigated in diabetes and associated with a successful pregnancy, they are worth-interesting candidates to be further evaluated in larger and clinically more heterogeneous cohorts to confirm their predictive role for the development of GDM.

Applications to Prognosis

In this chapter secreted frizzled-related protein 4 (sFRP4) and secreted frizzled-related protein 5 (sFRP5) have been reviewed in association with diabetes and gestational diabetes mellitus (GDM). The available studies show how sFRP4 and sFRP5 have an effect on insulin resistance and hyperglycemia, but a few studies used mainly *in vitro* models and animal studies and therefore it is interesting to evaluate whether these effects are also observed in human tissue. However, the increased sFRP4 levels and decreased sFRP5 serum levels are already associated with diabetes and GDM in human cohort studies. A few studies suggest that this differential expression of these proteins is already observed in the first trimester of the pregnancy while the onset, and diagnosis, of GDM is mainly observed in the late second or early third trimester (Schuitemaker et al. 2020; Oztas et al. 2016). Therefore, increased sFRP4 levels and decreased sFRP5 levels in the first trimester might be prognostic for the development of GDM. When sFRP4 and/or sFRP5 have a predictive power, a (therapeutic) intervention can be started or glycose levels can be monitored more intensively.

Applications to Other Diseases or Conditions

In this chapter secreted frizzled-related protein 4 (sFRP4) and secreted frizzled-related protein 5 (sFRP5) are associated with the development of gestational diabetes mellitus (GDM). Differential expression of sFRP4 is also associated with other obstetrical and gynecological disorders such as increased levels of sFRP4 in pre-eclampsia and decreased levels of sFRP4 in polycystic ovary syndrome (Zhang et al. 2013; Piltonen et al. 2013). The authors related this to possible progesterone resistance and predisposed reduced endometrial receptivity. An overlap of these markers in obstetrical disorders is more often seen and a possible reason for this is, for example, that preeclampsia and GDM seem to have a partially shared etiology (Odenkirk et al. 2020). In addition, GDM pregnancies also have an increased risk of preeclampsia and the disorders also share some common risk factors (McIntyre et al. 2019; Phipps et al. 2016). Next to obstetrical and gynecological disorders, sFRP4 is in literature related to various carcinoma, such as pancreatic ductal adeno, prostate, and colorectal carcinoma (Bernreuther et al. 2020; Busuttil et al. 2021; Natarajan et al. 2020; Huang et al. 2010). On the other hand, sFRP5 is more often related to cardiovascular and inflammatory diseases (Wang et al. 2020; Tong et al. 2019). Just like in diabetes, the predominantly inverse association of sFRP5 with the disorders is observed. sFRP5 is not as clearly associated in literature with pregnancy disorders as sFRP4, but it is worth mentioning that decreased sFRP5 was also correlated with the metabolic inflammation in polycystic ovary syndrome and elevated sFRP5 levels were observed during preeclampsia (Zhang et al. 2021, 2022).

Mini-Dictionary of Terms

- **Gestational diabetes mellitus**. The detection of a *de novo* hyperglycemia during pregnancy, generally detected in the late second trimester or early third trimester and resolves after birth.
- **Wnt signaling pathway**. The Wingless and Int-1 (Wnt) signaling pathways consist of three signal transduction pathways and they are involved in various biological processes, such as carcinogenesis, embryonic development, and cell proliferation.
- **Wnt/beta-catenin pathway**. This Wnt pathway does involve β-catenin and activation results in translocation of β-catenin into the nucleus and after interaction with T cell factor (TCF), β-catenin-related genes are transcripted.
- **Wnt/noncanonical pathways**. These Wnt pathways do not involve β-catenin and consist of the Wnt/Ca^{2+} and Wnt/planar cell polarity pathways. Among other processes, they regulate calcium release from the endoplasmic reticulum to control intracellular calcium levels, activate C-Jun N-terminal kinase (JNK), polymerize actin, and regulate transcription of pro-inflammatory factors.
- **Secreted frizzled-related proteins**. A protein family which are mainly antagonists of the Wnt signaling pathway through binding the agonist of the frizzled-related receptors and/or blocking the frizzled receptors by binding the receptor.

Key Facts of Secreted Frizzled-Related Proteins 4 and 5: What They Are and Can They Be Used as a Biomarker in Gestational Diabetes Mellitus

Key Facts of Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is one of the most common pregnancy-related disorders.

There is no worldwide agreement when to screen for GDM and its diagnostic definition and, therefore, worldwide prevalence rates are ranging from 1 to > 30%.

Most pregnant women get tested for GDM at 24–28 weeks of pregnancy.

Major risk factors for testing GDM are maternal age, body mass index, ethnicity, and family history of diabetes.

Short-term consequences of GDM are macrosomia (i.e., excessive weight gain of the fetus), increased risk of cesarian section, development of preeclampsia, and neonatal hypoglycemia.

Long-term consequences of GDM are primarily increased risk of developing T2DM, metabolic syndrome, and cardiovascular diseases for mother and T2DM, obesity, and cardiovascular diseases for the offspring later in life.

Key Facts of Secreted Frizzled-Related Proteins (sFRPs)

sFRPs consist of five extracellular glycoproteins and are antagonists of the Wingless and Int-1 (Wnt) signaling pathways.

All five members are approximately 300 amino acids long and contain a cysteine-rich domain, which is responsible for their antagonistic property in the Wnt pathway.

The Wnt signaling pathway consists of the canonical Wnt pathway and two noncanonical pathways: the planar cell polarity pathway and calcium pathway.

Activation of the canonical Wnt pathway results in gene transcription through translocation of β-catenin to the nucleus.

The Wnt/planar cell polarity pathway, also called C-Jun N-terminal kinase (JNK) pathway, regulates the cytoskeleton and activation of JNK.

The Wnt/calcium pathway regulates the calcium inside the cell through calcium release from the endoplasmic reticulum, activation of downstream kinases, and transcription of pro-inflammatory genes.

The Wnt signaling pathways are involved in various biological processes, such as embryonic development and cell differentiation, but also carcinogenesis and diabetes.

Summary Points

- Gestational diabetes mellitus is the detection of a *de novo* hyperglycemia during pregnancy, generally detected in the late second trimester or early third trimester and resolves after birth.

- Secreted frizzled-related protein 4 (sFRP4) and secreted frizzled-related protein 5 (sFRP5) are two antagonistic proteins for the Wingless and Int-1 (Wnt) signaling pathways and both are possibly involved in the development of diabetes.
- In both diabetes and GDM increased levels of sFRP4 and decreased levels of sFRP5 are observed before and during the disease.
- sFRP4 and sFRP5 are also related to the implantation and development of the placenta and their differential expression is associated with other pregnancy-related disorders, such as reduced placental growth and preeclampsia.
- sFRP4 plays a role in the glucose metabolism by suppressing the exocytosis of insulin of pancreatic beta cells.
- Second, sFRP4 increases the insulin-stimulated hepatic lipogenesis and hepatic insulin resistance by reducing insulin receptor substrate-1 (IRS-1) and forkhead box protein O1 (FOXO1).
- Decreased sFRP5 levels are responsible for reduced insulin sensitivity through blocking intracellular insulin receptor substrate-1 (IRS-1).
- Second, decreased sFRP5 levels will result in more activation of the noncanonical Wnt5A pathways and this pathway is associated with pro-inflammatory reactions.
- Both sFRP4 and sFRP5 are well investigated in diabetes but their role during pregnancy to a lesser extent and, therefore, these proteins are interesting candidates to be further evaluated in larger and clinically more heterogeneous cohorts to confirm their (predictive) role for the development of GDM.

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Insights into the World of MicroRNAs

31

miR-1281, a Novel Biomarker of Diabetic Retinopathy

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Contents

Introduction 650
miRNA Biogenesis 652
Circulating miRNAs 657
Circulating miRNAs and Diabetes 658
Circulating miRNAs and DR 659
miR-1281 as a Novel Circulating Biomarker in Patients with DR 660
Conclusions 662
Key Facts of miRNAs 663
Summary Points 664
References 664

Abstract

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes mellitus and the leading cause of vision loss in middle-aged, economically active people. So far, many modifiable and unmodifiable factors have been implicated in the pathogenesis of DR. Among them, microRNAs (miRNAs) – small non-coding RNA molecules that regulate gene expression – have shown to play an important role. Recent studies have identified differentially expressed circulating miRNAs in diabetic patients with and without DR, suggesting their potential use as noninvasive tools to early detect or predict DR progression. Although the actual knowledge is limited, understanding the molecular mechanism(s) linking miRNAs to DR could lead to novel diagnostic

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and therapeutic approaches for this invalidating complication of diabetes. The present chapter aims to summarize the role of miRNAs, and in particular of miR-1281, in the pathogenesis of DR and their promising use as disease-related biomarkers.

Keywords

Diabetic retinopathy · Circulating biomarkers · Angiogenesis · MicroRNAs · miR-1281

Abbreviations

AGEs	Advanced glycation end products
AGO	Argonaute
ARPE-19	Human retinal pigment epithelial cells
DGCR8	DiGeorge syndrome critical region 8
DR	Diabetic retinopathy
EPAC	Exchange protein directly activated by cAMP
HIF	Hypoxia-inducible factor
HIF1AN	Hypoxia-inducible factor-1α inhibitor
HUVEC	Human umbilical vein endothelial cells
Luc	Luciferase
miRNAs	MicroRNAs
PKA	Protein kinase A
PKC	Protein kinase C
RAAS	Renin-angiotensin-aldosterone system
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
TRBP	TAR RNA binding protein
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Introduction

MicroRNAs (miRNAs) are an evolutionarily conserved class of short (~19–22 nucleotides) noncoding single-stranded RNAs generated from genomic DNA (Bartel 2004), which modulate several important cellular processes, including tissue growth and development (Johnston and Hobert 2003; Zhao et al. 2005), cell proliferation and differentiation (Cheng et al. 2005; Chen et al. 2006), glucose metabolism (Chiefari et al. 2021), apoptosis (Cheng et al. 2005), senescence (Inukai and Slack 2013), and immune response (Chiefari et al. 2021). Consistent with these observations, miRNA dysregulation is widely involved in the pathophysiology of a number of complex human diseases, such as obesity, type 2 diabetes mellitus and related cardiovascular disease, cancer, and neurodegenerative disorders (Ardekani and Naeini 2010).

In general, miRNAs are expressed in tissue-specific patterns and exert negative posttranscriptional regulation. By binding target sites in the 3′ untranslated region (3′ UTR) of mRNAs, these molecules may act as gene silencers and can induce the degradation of protein-coding transcripts through the recruitment of deadenylases and decapping factors. On the other hand, miRNAs can also interact with other regions of the gene, including 5′ UTR, promoters, and coding sequences, so that may also affect gene transcription (Broughton et al. 2016).

The detailed mechanisms by which miRNAs regulate posttranscriptional expression of target genes need to be explored further. According to the current literature, mature miRNAs shuttle between nucleus and cytoplasm, at which levels their regulatory activities on transcription and/or translation of target genes occur (Makarova et al. 2016). Certain miRNAs are also released into the extracellular space and into biological fluids and taken up by recipient cells, often located distantly from the released sites, thereby acting like chemical messengers or hormones that mediate cell-cell communication and interactions (Makarova et al. 2016). The discovery of stable, extracellular miRNAs in blood plasma and serum samples, known as circulating miRNAs, has generated great interest in their potential use as noninvasive biomarkers for the identification and monitoring of diseases and related complications (Cortez et al. 2011). Recent investigations in this context suggest that, different from other RNA species, circulating miRNAs are highly resistant to RNase-mediated degradation, given that they are able to migrate in packed microvesicles and other intercellular structures that protect miRNA molecules from the endogenous RNase activity of the extracellular environment. Aberrant expression of circulating miRNAs has been linked to the development of many human tumors, as well as to the pathogenesis of common cardiovascular, neurodegenerative, and metabolic diseases, including type 2 diabetes (Chiefari et al. 2021; Ardekani and Naeini 2010). Furthermore, the dysregulation of specific miRNAs could take part in the inflammatory and endothelial dysfunctions that are associated with the occurrence of vascular complications in patients with diabetes (Zhang et al. 2017).

From what is known, type 2 diabetes mellitus is a complex form of diabetes that is characterized by chronically high levels of blood glucose, in which peripheral insulin resistance and insulin secretory dysfunction of the pancreatic beta cell are the major metabolic defects. If uncontrolled by lifestyle changes and pharmacological interventions, chronically sustained hyperglycemia may result in the development of multisystemic chronic complications, including microvascular (i.e., retinopathy, nephropathy, and neuropathy) and macrovascular complications (i.e., stable angina, acute coronary syndrome, cerebrovascular events, and peripheral arterial disease), which are associated with high morbidity and mortality in affected individuals (Mirabelli et al. 2019a, b; Palella et al. 2020). Today, type 2 diabetes is certainly well recognized as a major global health concern. It is estimated that by 2030 more than half a billion people worldwide will suffer from diabetes, with huge economic costs and negative impact on human health and well-being (Shaw et al. 2010). The accumulation of body fat, especially visceral fat, is negatively correlated with peripheral insulin sensitivity, and obesity is regarded as a key risk factor for type 2 diabetes and other conditions related to obesity, including the metabolic syndrome, which increases the risk of type 2 diabetes and coronary heart disease (Mirabelli et al. 2021a, b; Arcidiacono et al. 2020).

Diabetic retinopathy (DR) is among the most shattering microvascular complications of diabetes mellitus, as it may cause severe vision impairment and blindness in affected patients (Yau et al. 2012). Epidemiological studies have established that the increase in DR risk is particularly influenced by the duration of diabetes and by the efficacy of glycemic control (Yau et al. 2012). Also, as an established microangiopathic lesion of diabetes, DR may cluster with other traditional complications of diabetes, such as diabetic nephropathy and diabetic neuropathy, in which prolonged exposure to hyperglycemia is now recognized as the primary factor causing vascular damage in diabetes (El-Asrar et al. 2001).

The application of noninvasive biomarkers for the early detection of type 2 diabetes and identification of patients who are most at risk of complications should greatly improve diabetes management and care, as targeted interventions by clinicians might prevent or limit patients' vision loss and vascular damage. Recently, within the diabetes scientific community, much attention has been focused on circulating miRNAs as biomarkers for type 2 diabetes and its complications, and some of the miRNAs discovered so far in this field have even the potential for future therapeutic applications. In this regard, certain miRNAs have been identified to be involved in the neovascularization and augmentation of vascular permeability in retinal vessels by impinging on the vascular endothelial growth factor (VEGF) signaling pathway whose abnormal activation is a crucial event in DR and vascular damage (Chiefari et al. 2016). However, to date, only a few studies have investigated the progressive changes in circulating miRNA levels in patients destined to develop DR, using a longitudinal prospective design (Gong and Su 2017). Furthermore, sample populations in cross-sectional studies have often been of limited size, and some findings have not been replicated in other investigations. Clinical trials and observational investigations in large multiracial cohorts and standardization of circulating profiling technologies (Marzi et al. 2016) are now almost indispensable requirements to advance our knowledge on this topic and provide robust evidence for causal associations between miRNA biomarkers and the risk of DR. Table 1 summarizes the main results of human and preclinical studies addressing the potential roles and applications of aberrant circulating miRNAs in DR (Liu et al. 2019; Chen et al. 2017a; Jiang et al. 2017; Gomaa et al. 2017; Zou et al. 2017; Chen et al. 2017b; Santovito et al. 2021; Smit-McBride et al. 2020; Liu et al. 2018; Greco et al. 2020).

On the basis of these premises, in this chapter, we provide a brief overview of circulating miRNA biogenesis and functions and describe how miR-1281, a circulating miRNA signature for DR, has been identified and characterized by our group as an early biomarker of DR in patients with type 2 diabetes (Greco et al. 2020).

miRNA Biogenesis

The first miRNA, named lin-4, was discovered in the nematode worm *Caenorhabditis elegans* in 1993 (Lee et al. 1993). Since then, there has been a molecular biological revolution, and many of the components that participate in miRNA biogenesis and the basic principles of miRNA functions have been brought

Table 1 Selection of studies addressing the role and applications of circulating miRNAs in DR

Extracellular miRNA	Study type	Specimen(s)	DR-related expression change	Results	Ref.
miR-126	Cross-sectional study[a] (50 patients with proliferative DR and 30 controls with idiopathic macular hole)	Vitreous humor and blood plasma	Upregulated	Expression of miR-126 was inversely correlated with angiogenic factors (i.e., VEGF). Upregulation of circulating miR-126 was found in proliferative DR patients relative to nondiabetic controls	(Liu et al. 2019)
miR-21	Preclinical study (diabetic *db/db* mouse)	Mouse retina	Upregulated	In the *db/db* mouse model of diabetes, retinal upregulation of miR-21 related with reduced levels of PPARα, probably due to protein translation-inhibitory activities	(Chen et al. 2017a)
	Cross-sectional study[a] (65 diabetic patients without DR, 73 patients with nonproliferative DR, 51 patients with proliferative DR, 115 normoglycemic controls)	Blood plasma	Upregulated	Circulating mir-21 levels were positively correlated with DR severity and influenced by glycemic indices	(Jiang et al. 2017)
miR-200b	Cross-sectional study[b] (30 patients with proliferative DR and 29 controls with idiopathic macular hole)	Vitreous humor	Upregulated	Both miR-200b and VEGF levels were increased in the vitreous humor of patients with proliferative DR, but without significant correlation between them. miR-200b may be involved in the pathogenesis of proliferative DR through VEGF-independent mechanisms	(Gomaa et al. 2017)

(continued)

Table 1 (continued)

Extracellular miRNA	Study type	Specimen(s)	DR-related expression change	Results	Ref.
miR-93	Cross-sectional study[a] (75 patients with DR, 65 diabetic patients without DR, 127 normoglycemic controls)	Blood plasma	Upregulated	Upregulation of mir-93 related with the presence of DR, as well as with a longer duration of diabetes, glycemic indices, and circulating inflammatory markers (i.e., TNF-α, VEGF)	(Zou et al. 2017)
miR-146a	Preclinical study (transgenic mouse)	Mouse retina	Downregulated	miR-146a was downregulated in retinal tissue of diabetic wild-type mice, causing the production of inflammatory cytokines and extracellular matrix proteins. Overexpression of mir-146a in in vitro cultured endothelial cells and in diabetic transgenic mice prevented glucose-induced injury and inflammation	(Chen et al. 2017b)
miR-25-3p ***miR-320b***	Cross-sectional study[a] (20 patients with DR, 10 diabetic patients without DR, 10 normoglycemic controls)	Blood plasma	Upregulated	Higher levels of circulating miR-25-3p and miR-320b related with DR and DR severity. A possible biological relevance of these miRNAs in metabolic processes and endothelial cell proliferation was suggested by bioinformatic gene ontology analysis	(Santovito et al. 2021)

miR-495-3p	Cross-sectional study[a] (20 patients with DR, 10 diabetic patients without DR, 10 normoglycemic controls)	Blood plasma	Downregulated	Lower levels of circulating miR-495-3p related with DR and DR severity. A possible biological relevance of this miRNA in metabolic processes and endothelial cell proliferation was suggested by bioinformatic gene ontology analysis	(Santovito et al. 2021)
Let-7b, miR-320b, mir-762, and miR-4488	Cross-sectional study[b] (16 patients with DR, 10 controls with idiopathic macular holes or other non-diabetes related retinopathies)	Aqueous humor, vitreous humor, and blood plasma	Upregulated or downregulated depending on the bodily fluid type	DR related with altered expression of circulating let-7b, miR-320b, miR-762, and miR-4488. Each of these miRNAs could be either upregulated or downregulated in a unique manner, depending on the kind of body fluid	(Smit-McBride et al. 2020)
miR-211	Cross-sectional study (40 patients with DR, 40 diabetic patients without DR, 40 normoglycemic controls)	Blood serum	Upregulated	Among several differentially expressed circulating miRNAs, upregulation of miR-211 specifically related with DR	(Liu et al. 2018)
	Preclinical study (diabetic rats)	Rat retina	Upregulated	Upregulation of miR-211 related with vessel proliferation and reduced levels of SIRT1 proteins in retinal tissue of diabetic, insulinopenic rats	(Liu et al. 2018)

(continued)

Table 1 (continued)

Extracellular miRNA	Study type	Specimen(s)	DR-related expression change	Results	Ref.
miR-1281	Two-step cross-sectional study[a] (miRNA profiling step: 5 male patients with nonproliferative DR and 5 matched diabetic patients without DR; individual validation step: 20 patients with nonproliferative DR; 30 diabetic patients without DR)	Blood serum	Upregulated	Among several significantly upregulated circulating miRNAs in DR, miR-1281 emerged as the one being not influenced by other potential factors (i.e., gender, age, duration of diabetes). Extracellular release of miR-1281 by retinal epithelial cells, exposed to high glucose, stimulated the proliferation and migration of endothelial cells by interfering on the expression of VEGFA and activation of the HIF-1α pathway	(Greco et al. 2020)

[a]Type 2 diabetes mellitus was the cause of DR; type 1 diabetes mellitus was the cause of DR
[b]Enrollment criteria included both types of diabetes

to light. miRNAs are known to be transcribed from genomic DNA sequences into primary transcripts termed "pri-miRNAs," which are then processed into precursor miRNAs (pre-miRNAs), and finally mature miRNAs (O'Brien et al. 2018). However, it is worth mentioning that the current model for miRNA biogenesis comprises both canonical and alternative routes. In the predominant canonical route, miRNAs are processed from parental pri-miRNA transcripts by a cleavage mechanism involving two members of the RNase III family of enzymes, Drosha and Dicer, with a specific subcellular location (nuclear and cytoplasm, respectively) (Okada et al. 2009). Within the nucleus, Drosha endonuclease, in association with the RNA-binding protein DGCR8, cleaves the pri-miRNA into pre-miRNA that is then exported by Exportin 5 to the cytoplasm, where it is cleaved further by Dicer endonuclease, in association with its RNA-binding partner, TRBP, giving rise to mature double-stranded miRNA duplexes with the length of ~20 nucleotide (Zhang et al. 2004). The small RNA duplex is loaded into an Argonaute (AGO) protein, which is a key factor in the nuclear assembly of the RNA-induced silencing complex (RISC), and executes its effects by base-pairing with target mRNA. Maturation of RISC occurs following a process termed "strand selection" during which miRNA duplexes unwind and one of the two strands, the so-called guide strand, is incorporated in the mature RISC, whereas the other strand "passenger strand" of miRNA is usually degraded (O'Brien et al. 2018). Based on the direction of the starting filament, each RNA duplex can generate two miRNAs; the 5p strand originates from the 5′ end of the pre-miRNA hairpin, whereas the 3p strand is generated from the 3′ end. In addition to this classical pathway, many non-canonical miRNA biogenesis pathways have been described which are independent from Drosha or Dicer (Bogerd et al. 2010). As an example of miRNAs originating from non-canonical biogenesis pathways, mirtrons are a type of non-canonical miRNAs that mature through alternative steps that bypass Drosha processing and cleavage (Ruby et al. 2007). In this case, the intermediate pre-miRNA – defined by the entire length of the intron sequence from which it has origin – is excised by splicing and subsequently linearized by a debranching enzyme. This processing pathway leads to the generation of small RNAs that can repress matched targets, and there is evidence that this function can be mediated at least in part via the RNA-induced silencing complex effector AGO1. Therefore, as already pointed out (Ameres and Zamore 2013), miRNAs of mirtron origin may well represent an alternate source of miRNA-class regulatory RNAs (Ameres and Zamore 2013).

Circulating miRNAs

The first evidence for the presence of miRNAs in the extracellular environment was provided in 2008 (Mitchell et al. 2008). After this initial discovery, circulating miRNA molecules were detected in multiple biological fluids, including blood plasma and serum (Chen et al. 2008; Arroyo et al. 2011), cerebrospinal fluid (Cogswell et al. 2008), saliva (Gallo et al. 2012), breast milk (Zhou et al. 2012), and urine, and proved to show specific (in spatial and/or temporal terms) expression

signatures in response to both physiological and pathological stimuli (Weber et al. 2010). Soon it became clear that extracellular miRNAs have hormone-like behaviors, thereby modulating the activity of target recipient cells in either autocrine, paracrine, or endocrine manners (Iftikhar and Carney 2016). A large part of extracellular miRNAs are secreted in the extracellular fluid and transported to target cells within microvesicles, such as exosomes and apoptotic bodies (Gallo et al. 2012), or via binding proteins, including AGO2 protein (Arroyo et al. 2011) and high- and low-density lipoproteins (Vickers et al. 2011). It is now widely accepted that both membrane-enclosed microvesicles and miRNA-protein complexes ensure miRNA resistance to the action of extracellular ribonucleases, thus preventing them from premature degradation and shortened lifespan. Apoptotic bodies are the largest type of microparticles (0.5–2 μm) vehiculating miRNAs, which derive from cells undergoing apoptotic death (Thum and Condorelli 2015). The mechanism(s) for the uptake of miRNAs encapsulated in extracellular vesicles or conjugated to protein complexes by recipient cells are not completely clear, but several possibilities have been proposed including endocytosis, micropinocytosis, phagocytosis, or direct fusion of microparticles with plasma membranes. Also, some miRNAs can even interact with cell surface receptors on the recipient cells (Xu et al. 2013), thus acting with a hormone-like mechanism. Altogether these findings suggest that circulating miRNAs might play an important role as mediators of intercellular communications and may regulate critical mRNA targets and gene programs even in cells distantly located from their site of production (Rayner and Hennessy 2013). Owing to the high stability of miRNAs in mammalian biological fluids, and their efficient recovery and analysis by quantitative real-time PCR (qRT-PCR) and microarray platforms, the potential of circulating miRNAs as biomarkers for both diabetes monitoring and early identification of diabetes-related microvascular complications, including DR, has aroused great interest among researchers and clinicians working in this field.

Circulating miRNAs and Diabetes

Chronic sustained hyperglycemia in diabetes mellitus plays a major role in blood vessel injury and inflammation, as it may increase the production of advanced glycation end products (AGEs) (Brownlee et al. 1988), the activation of protein kinase C (PKC) and polyol pathways, and the production of reactive oxygen species (ROS) (Sima 2003), along with abnormal activation of the renin-angiotensin-aldosterone system (RAAS) (Gilbert et al. 2003). Extracellular miRNAs may not only contribute to a more accurate assessment of the metabolic profile in diabetes mellitus but may also participate to physiological processes in tissues or organs, such as the blood vessels, brain, kidney, and heart, that are negatively affected by chronically elevated blood glucose. Also, some miRNAs appear to be specific for certain selected forms of diabetes and are involved in the regulation of pancreatic beta-cell insulin production and secretion (Karolina et al. 2011; Poy et al. 2004; Tang et al. 2009), as well as in glucose utilization by the insulin-sensitive tissues skeletal muscle, liver, and adipose tissue (Chiefari et al. 2021).

The potential application of miRNAs in diabetes mellitus is strengthened by numerous recent studies, including the report by Chen et al. (Chen et al. 2008), demonstrating that circulating miRNAs with significantly different expression profiles were selected through the Illumina/Solexa sequencing method in patients with type 2 diabetes compared to normal glucose-tolerant subjects. After that, by using the microarray technology, Zampetaki et al. identified a characteristic signature of serum miRNAs (namely, miR-15a, miR-28-3p, miR-29b, miR-126, and miR-223), whose expression was downregulated in normoglycemic individuals who went on to develop type 2 diabetes over a 10-year period (Zampetaki et al. 2010). Likewise, in a subsequent study, the upregulation of seven miRNAs (namely, miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) was observed in patients with overt type 2 diabetes, when compared with subjects with prediabetes (Kong et al. 2011). Other studies in more recent years have focused on circulating miRNA profiling. However, it seems that the results obtained are not consistent. Certainly, what deserves a special attention in this research context is the fact that some circulating miRNAs are deregulated in individuals with impaired glucose tolerance, compared with people with normal glucose tolerance, even before the onset of overt type 2 diabetes, thus suggesting that variations in serum miRNAs may indeed reflect the progressive worsening of glycemic status. In particular, by comparing the plasma expression profiles of miRNAs between patients with newly diagnosed type 2 diabetes and patients with prediabetes, Yan et al. provided evidence that miR-1249, miR-320b, and miR-572 may serve as novel potential biomarkers for the diagnosis of type 2 diabetes in its early presymptomatic stage (Yan et al. 2016). Among miRNAs that have been reported to have a role in the pathogenesis of diabetes and its complications, miR-126-3p is the most well documented. miR-126 has been classified as an angiogenic miRNA or "angiomiR" because of its involvement in vascular regeneration and mobilization of hematopoietic stem/progenitor cells. This is one of the first miRNAs whose plasma levels have been found to be significantly reduced both in patients with type 2 diabetes and in patients at high risk of type 2 diabetes, suggesting miR-126 as a potential biomarker to predict type 2 diabetes in susceptible individuals (Zhang et al. 2015).

Circulating miRNAs and DR

As a leading cause of vision loss among working-age adults, DR is often the first microvascular complication of diabetes. It is also well known that patients diagnosed with DR may face an increased risk of other micro- and macrovascular complications of diabetes, when compared to subjects without DR, and, thus, the decline in life quality and expectancy can be expected to be fairly rapid in these individuals (Kramer et al. 2011). The pathogenesis of DR involves a variety of complex mechanisms related to inflammation, oxidative stress, and aberrant angiogenesis. Although limited, there is evidence that miRNAs may play a fundamental role during the early stages of DR, including endothelial cell injury, vascular leakage, vessel growth, and oxidative stress response of retinal cells to glucose stimuli. Most

of the studies addressing specific miRNAs and their role in DR are in vitro studies, which refer to retinal tissues from animal models or to endothelial cells exposed to high glucose conditions to mimic a diabetic environment (Mortuza et al. 2014; Yan et al. 2015; McArthur et al. 2011). Up to now, only a few studies have analyzed DR-related miRNAs in human specimens, particularly from patients with type 1 diabetes mellitus. In a groundbreaking clinical research study published in 2016, a panel of 29 candidate serum miRNAs, previously defined as diabetes-related, was tested in 2 independent cohorts of matched patients with type 1 diabetes, with and without nonproliferative DR at baseline. Two angiogenic miRNAs, miR-27b and miR-320a, were identified as potential biomarkers for new-onset DR and progression of DR (Zampetaki et al. 2016). It is worth emphasizing, however, that dysregulated miRNAs associated with DR do circulate not only in blood but also in the vitreous and aqueous humor of the eye (Ragusa et al. 2013). Among miRNAs mediating oxidative stress-related tissue damage in DR, miR-7, miR-15a, miR-27b, miR-100, miR-145, miR-195, miR-200b, miR-365, miR-383, and miR-455–5p were identified as the most relevant (Li et al. 2020). In particular, Garcia-Morales et al. have shown that experimental exposure to hypoxic environment increased the expression of miR-7 in human umbilical vein endothelial cells and mouse retina, leading to vascular hyperpermeability (Garcia-Morales et al. 2017). In line with this, as demonstrated by multiple authors, including our own group, hypoxia can increase oxidative stress and damage endothelial intercellular adhesion by affecting key signaling pathways that are activated in response to hypoxia-inducible factors (HIFs) and/or non-coding RNAs (i.e., protein kinase A, PKA, pathway, and exchange protein directly activated by cAMP, EPAC, pathway) (Garcia-Morales et al. 2017; Arcidiacono et al. 2017; Messineo et al. 2016).

In 2019, by using a high-throughput RNA-Seq analysis, Li et al. evidenced that miR-4448, miR-338-3p, miR-190a-5p, miR-485-5p, and miR-9-5p were differentially expressed in serum from nonproliferative DR patients and type 2 diabetic patients without DR (Li et al. 2019). However, the limited sample size and failure of patient matching on some clinical variables (i.e., age and gender) may have affected, at least in part, the lack of research reproducibility in these studies. So far, no circulating miRNA has been unequivocally linked to DR, so that we are still far from a consensus on the extent to which miRNA signatures may serve as predictors of diabetic complications. The heterogeneity of methodologies employed in these studies to detect circulating miRNAs and the different diagnostic criteria adopted for DR, along with variations in disease severity at the time of ophthalmological examination, may explain at least part of this lack of agreement.

miR-1281 as a Novel Circulating Biomarker in Patients with DR

In 2020, by adopting a two-step cross-sectional study design, complemented by in vitro experiments, we developed an original research approach aimed at identifying circulating miRNAs differentially expressed in patients with type 2 diabetes, with and without DR, and at evaluating their predictive and pathogenic role for this

diabetic complication (Greco et al. 2020). In the first part of the cross-sectional study, fasting serum samples from ten randomly selected matched male patients with type 2 diabetes, either with or without nonproliferative DR (in the absence of other diabetic microvascular complications), were pooled and used for miRNA profiling. As differences in miRNA expression have been reported between the sexes, female patients were excluded to avoid the influence of sex steroid hormones, such as estrogens, on miRNAs (Sharma and Eghbali 2014). miRNAs were recovered from the aqueous phase of pooled sera by using a commercial silica-based column system (*miRNeasy* – Qiagen) and spiking in an exogenous, synthetic miRNA mimic (*Caenorhabditis elegans* miR-39) to allow for normalization and estimation of miRNA extraction and reverse transcription efficiency. By adopting an arbitrary threshold of at least twofold changes, we observed that, out of 372 potentially detectable miRNA on a pre-designed miRNA array (i.e., *miScript miRNA PCR Array Human Serum & Plasma 384HC* – Qiagen), 40 were significantly upregulated, and 3 were downregulated in pooled sera of DR patients with respect to non-DR patients. In an attempt to perform a more stringent selection of circulating miRNAs predictive of DR, only the ones showing at least fivefold changes were chosen for the second part of the study. In this further step, individual validation of the most relevant miRNAs was conducted by qRT-PCR in all study participants from an extended cohort of unrelated type 2 diabetic patients with nonproliferative DR ($n = 20$) and new age- and BMI-matched controls ($n = 30$). In this group of clinically well-characterized diabetic patients, the overexpression of five circulating miRNAs was found significantly correlated with early DR, and, among them, miR-1281 was identified as the best predictor of DR, as shown by multiple linear regression analysis and receiver operating characteristic (ROC) analysis. Thus, we focused our attention on this specific miRNA and performed extensive in vitro studies in which human retinal pigment epithelial cells (ARPE-19) and human umbilical vein endothelial cells (HUVEC) were exposed to standard (5 mM) or high (25 mM) glucose concentration and cell migration ability and VEGFA expression were measured after a 48-h incubation period. In both cell models, exposure to a condition of hyperglycemia, as the one mimicked by the addition of 25 mM glucose to the media, was able to increase the intracellular levels of miR-1281. Nonetheless, retinal and endothelial cells considerably differed in their behavior and responsiveness to glucose changes, given that secreted levels of miR-1281 were upregulated in the supernatant of high glucose-treated ARPE-19 cells, but not of HUVEC cells. In both cell lines, exposure to high glucose resulted in the upregulation of VEGFA mRNA and protein expression compared with low glucose-treated cells, thus suggesting that a link may indeed exist between diabetes-induced dysfunction of the retinal epithelial tissue, enhanced extracellular release of miR-1281, and hyperexpression of VEGFA, a mitogen which plays a role in DR, by inducing vascular permeability and neovascularization of retinal vessels. To investigate the plausibility of this pathogenetic hypothesis, we examined the effect of increasing amounts (0–250 μL) of conditioned medium enriched in ARPE-19-secreted miR-1281 on VEGFA expression in HUVEC endothelial cells. After 48 h of treatment under these conditions, we observed that VEGFA mRNA and protein expression increased in

HUVEC cells in a dose-dependent manner. Similar results were obtained by treating HUVEC cells with miR-1281 mimic (150 ng for 48 h), thereby consistently supporting the hypothesis that early vascular damage in patients with DR is mediated, at least in part, by the overexpression of miR-1281 and its involvement in the upregulation of VEGFA.

Next, to test whether the VEGFA gene promoter could be under the control of miR-1281, HUVEC cells were transiently co-transfected with the luciferase (Luc) reporter plasmid, VEGF 2.6-Luc, together with miR-1281 mimic. In these dual-luciferase assays, treatment of human endothelial cells with miR-1281 mimic was proven to induce a significant increase in VEGFA promoter-driven Luc activity, which was paralleled by an increase in VEGFA protein abundance, thereby indicating that miR-1281 activates VEGFA gene transcription.

How miR-1281 can orchestrate this complex scenario is not clear, and more investigation is needed before firm conclusion can be drawn. The *TargetScan* bioinformatic prediction algorithm revealed that miR-1281 could potentially target 3' UTR sequences of the hypoxia-inducible factor-1α inhibitor (HIF1AN), thereby accelerating its mRNA decay. The accumulation of HIF-1α due to miR-1281-induced degradation of HIF1AN would increase HIF-1α (a master regulator of hypoxia) and enhance the transcription of hypoxia-responsive genes, including *VEGFA*. Furthermore, assessment of cell migration by wound healing assays of HUVEC cell monolayer revealed that directional cell migration was increased in miR-128-treated cells, in which wound closure was enhanced relative to untreated control cells, thus supporting the postulated role of miR-1281 in promoting retinal endothelial dysfunction, which results in the development of DR (Fig. 1). Whether miR-1281 has a functional role in glucose-induced retinal damage by VEGFA via HIF-1α is yet to be proved. However, if our interpretation is correct, this would mean that miR-1281 is a novel pathogenetic biomarker of retinal microangiopathic damage in patients with diabetes, which may help in explaining how chronic hyperglycemia could abnormally induce VEGFA expression in retinal cells. It also has to be considered that, in addition to VEGFA, other downstream targets of HIF-1α, which are involved in the directional migration of retinal endothelial cells, such as adhesion molecules, might be involved as well (Olson et al. 1997). Whether the expression of these molecules can be affected by miR-1281 is an interesting issue that could be addressed in the future.

Conclusions

DR is a highly invalidating disease that is associated with enormous social and health burdens. The identification of novel noninvasive circulating biomarkers of DR, such as miR-1281, could potentially allow early diagnosis of this microvascular complication of diabetes mellitus and support programs of personalized surveillance in at-risk patients. Furthermore, the evidence for a pathogenetic role of miR-1281 would also sustain its potential as a target for future therapeutic strategies (i.e., the use of antagomirs). However, more investigation is needed to better characterize the

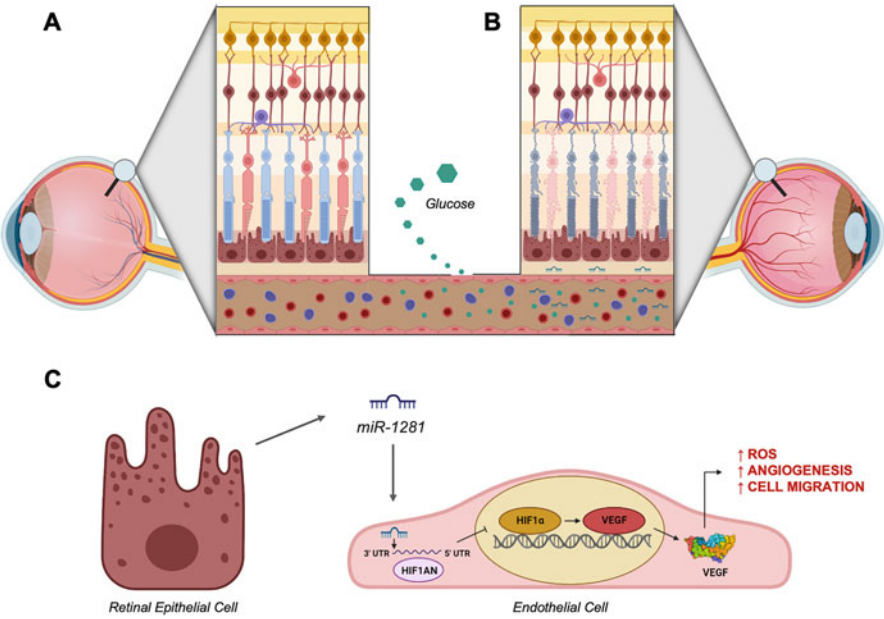

Fig. 1 Diabetic retinopathy and miR-1281. (**a**) Human eye structure with focus on the retina under normal conditions. (**b**) Retinal damage in response to the rise of blood glucose. (**c**) Postulated cross-talk between retinal epithelial and endothelial cells in DR: miR-1281 is released in the extracellular space by retinal epithelial cells exposed to hyperglycemia and binds to the 3′ UTR sequence of the HIF1AN transcript in endothelial cells, leading to the upregulation of both HIF-1α and VEGFA

molecular and functional relationship between miR-1281, HIF-1α, and VEGFA-related angiogenic pathways, whereas studies with larger cohorts of patients are required to validate the role of miR-1281 in a clinical setting. Finally, in order to improve research reproducibility and definitively establish circulating miRNAs as clinically reliable disease biomarkers, it becomes fundamental to standardize both sample collection and preparation procedures and the analytical methodologies in order to obtain comparable results.

Key Facts of miRNAs

- miRNAs are in control of production, processing, and/or degradation of target mRNAs and encoded proteins.
- Mechanistic functions of miRNAs can be assessed bioinformatically and/or in in vitro studies. A growing number of databases and algorithms (i.e., *TargetScan*) are available to predict miRNA-binding sites on protein-coding genes and their associated networks. Experimental validation of these putative miRNA-mRNA interactions is mandatory.

- miRNAs can be secreted in the extracellular fluid and transported to target cells within microvesicles or through binding proteins. Some miRNAs can even interact with cell surface receptors, thereby emerging as novel cellular messengers in endocrinology.
- Circulating miRNAs show high stability in biological fluids; therefore, they can be seen as potential biomarkers for type 2 diabetes and related diseases.
- Dysregulated miRNAs have a role in the pathophysiology of diabetes and diabetic microvascular complications. Their modulation may represent a novel therapeutic target.

Summary Points

- Patients with DR display an increased risk of morbidity and mortality from cardiovascular diseases and other diabetic microvascular complications.
- Extracellular miRNAs in blood plasma, serum, and ocular fluids from patients with DR may represent novel noninvasive biomarkers for the early identification and monitoring of this condition.
- Certain extracellular miRNAs involved in the pathogenesis of DR and other diabetic microvascular complications could induce vascular damage by modulating VEGFA expression and are thus termed "angiomiRs."
- Recent in vitro studies show that miR-1281 is released in the extracellular space by retinal cells in response to hyperglycemia and affects endothelial cell migration by regulating the HIF-1α/VEGFA signaling pathway.
- Elevated levels of miR-1281 in blood plasma appear to be specifically associated with DR in patients with type 2 diabetes.

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Genomic Ancestry as Biomarkers

32

Links with Diabetic Retinopathy

Deborah Conte Santos

Contents

Introduction 670
How to Determine Ethnicity? 672
How to Assess Genetic Ancestry? 672
Short Tandem Repeats (STRs) 673
SNPs 673
INDELs 673
AIMs 674
The Role of Ancestry in DR: What Do We Know so Far? 674
Conclusions 676
Applications to Other Diseases or Conditions 676
Mini-Dictionary of Terms 676
Key Facts of Genomic Ancestry as a Biomarker in Diabetic Retinopathy 677
Summary Points 677
References 677

Abstract

The influence of ethnicity on diabetic retinopathy has been studied for decades with controversial results. Different populations report different prevalence for diabetic retinopathy, demonstrating a possible correlation. With the advent of genetics, it has become clear that self-reported ethnicity does not correlate with genomic ancestry. The chapter goes on to discuss research in genomic ancestry (and ethnicity) and diabetic retinopathy.

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Keywords

Genomic ancestry · Ethnicity · SNPs · INDELs · AIMs · Type 1 diabetes · Type 2 diabetes · Microvascular complications · Diabetic retinopathy · Eye · Retina · Biomarker · Genetics

Abbreviations

ACE	Angiotensin-converting enzyme
AIMs	Ancestry informative markers
DNA	Deoxyribonucleic acid
DR	Diabetic retinopathy
GWAS	Genome-wide association study
INDELs	Insert/deletion polymorphism
NOS 3	Endothelial nitric oxide synthase
NPDR	Nonproliferative diabetic retinopathy
RAGE	Advanced glycation end-product receptors
SNPs	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor

Introduction

Diabetic retinopathy (DR) is one of the most feared chronic complications in type 1 and type 2 diabetes due to its potential for vision impairment or permanent vision loss. Besides being a preventable cause, DR is one of the leading causes of blindness in the working-age population (Leasher et al. 2016; Steinmetz et al. 2021). The prevalence of DR varies among different areas of the globe and ethnicities (Zhang et al. 2010; Federation 2019). A recent meta-analysis showed the lowest prevalence in Asia (16.9%) and South/Central America (13.3%) and higher prevalence in Africa (35.9%), North America/Caribbean (33.3%), and the Middle East/North Africa (32.9%), as shown in Fig. 1 (Teo et al. 2021). Differences in prevalence can also be found within the country. In the USA, prevalence can vary from 36.5% to 77.6% in type 1 diabetes and from 28.5% to 40.3% in type 2 diabetes (Lee et al. 2015). In China, the Northwest region has the highest prevalence of DR among the diabetes population (19.4%), while the East shows the lowest (17.7%) (Song et al. 2018). Several factors can explain those differences, such as the diagnosis criteria, the capacity of screening, and differences in ethnicity.

Patients with type 1 diabetes are more likely to present DR than patients with type 2 diabetes (Barometer 2017; Federation 2019). The global estimated prevalence in type 1 diabetes patients is 25%, while type 2 patients show a prevalence of 16% (Barometer 2017). A recent study in a youth population of type 1 and type 2 diabetes patients in the USA showed that T1D individuals had a higher prevalence of DR than

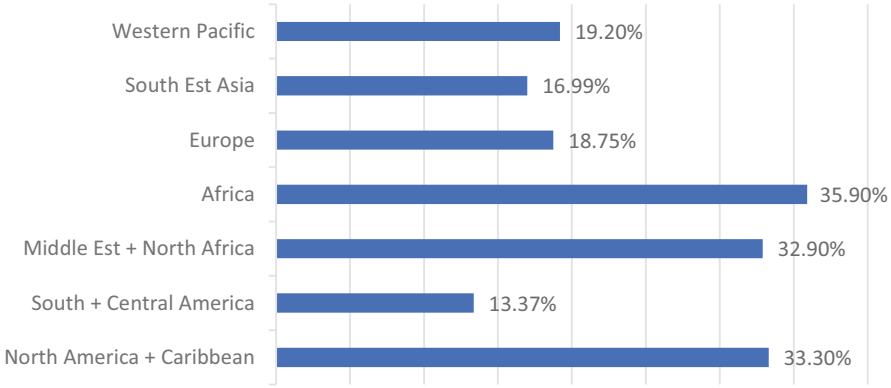

Fig. 1 Prevalence of diabetic retinopathy in different countries. (Teo et al. 2021)

T2D (20,1% vs. 7,2%, respectively). In addition, patients with T1D developed DR sooner than T2D patients (Wang et al. 2017). In Europe, the difference between type 1 and type 2 prevalence of DR is also present (54.4% vs. 25%, respectively) (Li et al. 2020).

Several large studies in different populations have well-established classic risk factors for DR (Sjølie et al. 1997; Stratton et al. 2001; Zheng et al. 2012; Gerstein et al. 2013; Melo et al. 2018; Hainsworth et al. 2019). Hyperglycemia, hypertension, and diabetes duration are the highest risk factors for developing DR. Therefore, achieving sustained good glycemic and blood pressure control is essential to prevent DR in type 1 and type 2 diabetes. However, those factors account for only 10% of the risk of developing DR (Klein et al. 1998). In clinical practice, it is often observed that some patients with long diabetes and inadequate glycemic control do not present any DR, and some patients develop early DR despite reasonable glycemic control. Therefore, other factors (genetics or environmental) have to be considered when accessing the risk for DR.

Multiple genetic markers have been studied in the last decade, some with controversial results. These heterogenic results might be explained by differences in the diagnosis criteria of DR, the type of methodology used, and differences in the ethnic background of the studied populations. Regarding the kind of methodologies, the most used are linkage analysis, candidate gene study, GWAS (genome-wide association study), and WES (whole-exome sequencing). As for positive association results, polymorphisms of the genes associated with oxidative stress and inflammation are the most common, such as AKR1B1, VEGF, RAGE, NOS3, and ACE (Simó-Servat et al. 2013). Other studied genes showed low reproducibility and weak associations (Cabrera et al. 2020).

Hence, the study of genetic biomarkers should be associated with studying the background ethnicity of the populations. In this chapter, we will discuss the role of genomic ancestry as a biomarker of DR.

How to Determine Ethnicity?

The determination of ethnicity based on self-reported color/race comes with many biases and, at some point, can acquire negative connotations and prejudice. Several studies have demonstrated that skin color cannot predict an individual genetic ancestry (Bryc et al. 2015; Batai et al. 2021).
Although self-reported color/race has been primarily used to determine ethnicity, with the advent of genetic methodologies, it is clear that self-reported color/race does not correlate with ancestry, especially in highly admixed populations. Also, the imprecise use of ethnicity might lead to misclassifications and impaired communications of genomic research results (Bonham et al. 2018).

A recent study on 9138 subjects referred to carrier screening identified discrepancies between self-reported ethnicity and genetic ancestry. Only one-third of the participants that presented Mediterranean genomic ancestry self-reported this on requisition forms. The highest degree of ancestral admixture was present in patients who self-reported Latin American (Shraga et al. 2017).

A study analyzed individuals' STR-Y chromosome and mitochondrial DNA ancestry markers in 120 participants self-declared as White and 50 as Brown. The results show a significant presence of Caucasian ancestry in both groups with the STR-Y chromosome analysis, while the mitochondrial DNA showed similar proportions of the three principal ancestries (Caucasian, African, and Amerindian) (Ferreira et al. 2006). In another study that compared self-reported race and mitochondrial DNA, they found a significant presence of African ancestry (37.6%) in the self-reported White group (Cardena et al. 2013).

A large study in Brazil showed high degrees of genomic ancestry diversity with the great contribution of the European ancestry, followed by the African and a low percentage of Amerindian ancestry (Lima-Costa et al. 2015).

Another study in Brazil with type 1 diabetes patients showed that even the group self-declared Black had a median proportion of European ancestry of almost 40%. In the same study, only 6.3% of the patients had a European contribution higher than 95% (Gomes et al. 2018).

In addition, several factors could influence self-reported ethnicity, including access to work, social background, and social policies (Paschetta et al. 2021). Therefore, public health key decisions such as drug administration, therapy design, and clinical trials should not be based exclusively on self-reported ethnicity. A survey with Brazilian students showed that self-classification of race varied across short intervals, indicating how the environment could influence it and how imprecise it can be (Santos et al. 2009). Therefore, the study of genetic markers for DR should include genomic ancestry analysis to better report the results and provide more external validation to their findings.

How to Assess Genetic Ancestry?

Genomic ancestry can be assessed basically by three different methodologies: short tandem repeats (STRs), single nucleotide polymorphisms (SNPs), and insertion-deletions (INDELs). The primary rationale presented by all methods is to estimate

the proportions of ancestry presented in the genome of the individuals, comparing them to an established reference population. Genomic ancestry can be used at an individual or population level and should account for population differences when reporting disease associations (Fujimura and Rajagopalan 2011). Nevertheless, genomic ancestry cannot be seen as a tool to determine an individual's ethnicity/race as a discrete category. The concept of an individual's ethnicity/race relates to several other determinants (social identity, socioeconomic status, health) that should be considered (Bonham et al. 2018).

Short Tandem Repeats (STRs)

The study of ancestry with STRs can analyze either maternal (mitochondrial DNA, mtDNA) or paternal inheritance (Y chromosome). The non-recombining portion of the Y chromosome (NRY) is transmitted practically intact through the paternal strains but is present only in males (Jobling and Tyler-Smith 2003). Mitochondrial DNA can be found in both males and females. It provides information on the direct female ancestral line as the mitochondria come exclusively from the mothers, and the mtDNA stays intact in the mitochondria during meiosis. Therefore, the Y chromosome and mtDNA marker analysis can reveal the individual's single ancestral lines and establish an ancestral population's mating pattern (Kayser 2017).

SNPs

Single nucleotide polymorphisms (SNPs) represent a replacement in a nucleotide in DNA sequencing and are the most common type of genetic variation. It is estimated that an individual's genome has, on average, four to five million SNPs. Some SNPs are linked to a specific ancestry/ethnicity and are used as a reference to estimate the proportion of different ancestries of a given individual by comparing the genotypes found (Sampson et al. 2011).

The development of genotyping technologies led to an increase in genome-wide association study (GWAS) (Pereira et al. 2012). GWAS uses high-density genotyped data of millions of polymorphism markers, allowing not only estimates of ancestry but also several associated disease biomarkers. Although very informative, this method is very costly. Due to limited funding in several countries, as an alternative, there are validated small sets of SNPs to predict ancestry accurately (Sampson et al. 2011).

INDELs

Insertion-deletions (INDELs) represent length changes/mutations in a genomic sequence that could be used to identify a specific population in the context of population genetics. INDEL genotyping is simple, requiring a PCR followed by capillary electrophoresis, whereas SNPs need more specific sequencing methods (Pereira et al. 2012).

AIMs

Ancestry informative markers (AIMs) are genetic markers that present substantially different frequencies between different populations (Phillips et al. 2007). They could be a single polymorphism, INDELs, or STRs from the Y chromosome. A group of AIMs can identify the genomic ancestry of an individual. The result comes from comparing the individual AIMs and the characteristic AIMs from a reference population, generating a spectrum of proportions of ancestries using a statistical probability modeling approach (Pritchard et al. 2000; Qu et al. 2012). The result, for example, of an individual's ancestry might be described as 10% Native American, 50% European, and 40% African. AIMs are specially used in the population genetics field to assess admixed population structure (Pereira et al. 2012). They are less expensive than GWAS or other dense SNP genotyping and could easily be interpreted and analyzed, becoming an essential tool in disease association studies.

The Role of Ancestry in DR: What Do We Know so Far?

The study of ethnicity and ancestry as a risk factor for DR shows conflicting results (Sivaprasad et al. 2012a). DR prevalence and progression vary within different ethnical groups (Emanuele et al. 2009; Lee et al. 2015). The vast majority of the studies do not separate between the types of diabetes and use different DR diagnosis criteria. Moreover, few studies were performed in admixed populations, and ethnicity is based on self-reported information.

A summary of the studies published in the last years and the type of information used to define ethnicity/ancestry are shown in Table 1. Only three studies used genomic ancestry to assess ethnicity in evaluating DR risk factors (Gao et al. 2014; Tandon et al. 2015; Santos et al. 2020). Two of them were performed in type 2 diabetes patients from two different cohorts in the USA, and the AIM-SNP genotyping method was used. The first one, in 944 type 2 diabetes Latinos (135 with severe DR and 809 with moderate, mild, or no DR), showed that Native-American ancestry was associated with severe DR (proliferative or severe nonproliferative DR) (Gao et al. 2014). The other studied a group of 305 African-Americans with PDR and 1135 controls with nonproliferative DR or no DR. In this study, African genomic ancestry was associated with PDR only in the univariate analysis but not after controlling for sociodemographic factors (Tandon et al. 2015). The third study was conducted in 414 Brazilian type 1 diabetes patients divided into 176 with severe DR (PDR or severe nonproliferative) and 238 without DR matched by diabetes duration. Genomic ancestry was assessed by 46-AIMs/INDELs. They found a positive association between African genomic ancestry and severe DR even after adjusting for sociodemographic variables (Santos et al. 2020).

Several studies reported positive associations between self-reported ethnicity and DR. A study from a South African population showed that the prevalence of DR was higher in Blacks compared to Indians (55.6 vs. 45.5%, respectively) and that the Blacks presented the onset of DR earlier than Indians (13.0 +/− 4.6 years vs. 18.0 +/− 4.6 years,

Table 1 Summary of published studies that analyzed ethnicity and diabetic retinopathy

Reference	Type of diabetes	Number of patients (N)	Country	Year	Ethnicity assessment	Results
Santos et al. (2020)	TD1	176 PDR or severe NPDR/ 238 no DR	Brazil	2020	AIMs/ INDELs	African genomic ancestry as a risk factor
Gao et al. (2014)	TD2	135 PDR or severe NPDR / 809 other DRs or no DR	USA	2014	SNPs	Native-American ancestry as a risk factor
Tandon et al. (2015)	TD2	305 PDR/1135 other DRs or no DR	USA	2015	AIMs/ SNPs	African ancestry as a risk for PDR, but no association after adjustments
Zhang et al. (2010)	TD1/ TD2	1006 diabetes patients	USA	2010	Self-reported	Non-Hispanic Blacks have higher prevalence of DR than non-Hispanic Whites
Varma et al. (2014)	TD1/ TD2	1038 diabetes patients	USA	2015	Self-reported	Non-Hispanic Blacks have higher prevalence of DME than non-Hispanic Whites
Raymond et al. (2009)	TD2	421 South Asians/614 White Europeans	UK	2009	Self-reported	South Asians have higher prevalence of DR than White Europeans
Wong et al. (2006)	TD1/ TD2	778 diabetes patients	USA	2006	Self-reported	Blacks and Hispanics are more prone to any DR than Whites, but no association after adjustments
Sivaprasad, Gupta et al. (2012b)	TD1/ TD2	50,285 diabetes patients	UK	2012	Self-reported	South Asians and Blacks are more likely to have visual impairments than Whites
Tan et al. (2018)	T1D/ TD2	2877 diabetes patients	Singapore	2018	Self-reported	Indian ethnicity as a risk factor for any DR (vs. Chinese)
Motala et al. (2001)	TD1/ TD2	219 diabetes patients	South Africa (Durban)	2001	Self-reported	Higher prevalence and early onset of DR in Blacks than Indians
Klein et al. (1994)	T1D	200 diabetes patients	USA	1994	Self-reported	Higher prevalence of DR in Whites compared to African-Americans

T1D, type 1 diabetes; TD2, type 2 diabetes; PDR, proliferative diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; DR, diabetic retinopathy; AIMs, ancestry informative markers; INDELs, insert/deletions; SNPs, single nucleotide polymorphism; DME, diabetes macular edema

$P < 0.05$) (Motala et al. 2001). Two studies from the USA demonstrated that non-Hispanic Blacks had a higher prevalence of DR and diabetic macular edema than Whites (Zhang et al. 2010; Varma et al. 2014). Another study on type 2 diabetes from the UK found that South Asians had a higher prevalence of DR than Whites (Raymond et al. 2009). In contrast, another study showed White ethnicity as a risk factor for the development and progression of DR compared to African-Americans (Klein et al. 1994).

Multi-ethnic cohorts including Whites, Blacks, South Asians, and Hispanics performed in the USA (Wong et al. 2006) and the UK (Sivaprasad, Gupta et al. 2012b) did not show an association between ethnicity and DR. Nevertheless, the UK cohort found that South Asians were more prone to visual impairment than Blacks. Moreover, the Singapore Epidemiology of Eye Diseases Study (Tan et al. 2018) demonstrated that Indians had a higher prevalence of DR than Chinese.

Conclusions

In conclusion, the study of DR biomarkers should consider the analysis of genomic ancestry to mitigate the lack of reproducibility found throughout the years. Moreover, genomic ancestry could be a risk factor per se. Future studies are needed to validate these findings and improve better screening for high-risk populations.

Applications to Other Diseases or Conditions

Genomic ancestry might be studied as an independent risk factor (biomarker) for other diabetic complications and diseases with a similar physiopathological base. Moreover, genomic ancestry applies to all genetic studies of several diseases and should be used to stratify the studied populations and improve the quality and homogeneity of reporting results.

Mini-Dictionary of Terms

- **Self-reported ethnicity**. *A categorization of race is that the individual sees himself as belonging to a specific race group. Cultural, economic, and social aspects can influence it.*
- **Genomic ancestry**. *Uses genetic markers to infer the proportions of ancestry lineages of an individual.*
- **Statistical probability modeling approach**. *This term refers to a statistical analysis that calculates the genetic distances of an individual ancestry marker to a reference population, generating the proportions of genomic ancestries that belong to that individual or population.*
- **Mitochondrial DNA**. *The mitochondria present in the human cell cytoplasm come primarily from the maternal egg. Mitochondria have genetic material that*

does not suffer a lot of mutation, being an important tool for genealogical research.

Key Facts of Genomic Ancestry as a Biomarker in Diabetic Retinopathy

- Ancestry informative markers (AIMs) are genetic markers that present substantially different frequencies between different populations.
- A group of AIMs can identify the genomic ancestry of an individual.
- They could be a single polymorphism, INDELs, or STRs from the Y chromosome.
- An individual's ancestry might be described as 10% Native American, 50% European, and 40% African.
- AIMs are specially used in the population genetics field to assess admixed populations' structure.

Summary Points

- The prevalence of DR varies with ethnicities from 13.3% (South/Central America) to 35.9% (Africa).
- There is a lack of reproducibility in the study of biomarkers for diabetic retinopathy.
- Self-reported race/ethnicity is not appropriate for reporting associations of genetic diseases as it does not correlate with genomic ancestry.
- In type 2 diabetes patients, Native-American genomic ancestry was associated with severe DR, whereas in type 1 patients, African genomic ancestry presented as a risk factor for DR.
- Regarding self-reported ethnicity studies, a wide range of results is reported (Blacks vs. Indians; Whites vs. African-Americans; South Asians vs. Blacks; Indians vs. Chinese).

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Hp1-1 as a Genetic Marker in Diabetes: Measures, Applications, and Correlations

33

Agnieszka Filipek

Contents

Key Facts of Haptoglobin Phenotype 683
Introduction 683
Structure and Polymorphic Variants of Haptoglobin 684
Biological Functions of Haptoglobin 685
Correlations of Haptoglobin 686
The Relationship Between the Haptoglobin Variant and Cardiovascular Disease 690
The Relationship Between the Haptoglobin Variant and the Development of Diabetic Complications 691
Relationship Between the Haptoglobin Variant and Neurodegenerative Diseases 692
The Relationship Between the Haptoglobin Variant and the Development of Infection 693
The Relationship Between the Haptoglobin Variant and the Development of Immune-Mediated Diseases 695
Conclusion 696
Summary Points 697
References 698

Abstract

In humans, the acute phase protein, haptoglobin, exists in two basic genotypes: Hp1 and Hp2, which gives three phenotypes: Hp1-1, Hp2-1, and Hp2-2. Homozygous individuals possess type 1 alleles encoding Hp and are characterized by high expression of Hp1-1. The Hp2-2 phenotype is present in homozygous individuals who only have type 2 alleles. Heterozygous individuals have type 1 and type 2 alleles, which is associated with high expression of Hp2-1. The structural structure of haptoglobin chains determines its properties and functions. Hp1-1 short chains easily imply complexes with hemoglobin and enhance the production

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of anti-inflammatory markers, i.e., CD163, IL-10, and HO-1. Consequently, Hp1-1 plays a protective role by reducing oxidative stress and chronic inflammation in the body. In contrast, Hp2-2 is a long-chain protein which makes it difficult to complex hemoglobin. Free hemoglobin increases the production of ROS, which increases inflammation, which can lead to the development of many diseases, including complications in people with type 2 diabetes, such as cardiovascular disease, retinopathy, and nephropathy. It has been suggested that the Hp2-2 phenotype is also associated with the increase in bacterial and viral infections, which are one of the causes of higher mortality in people with diabetes. In this review, we also demonstrated an ambiguous role for haptoglobin in, for example, neurodegenerative diseases where Hp2-2 appears to be protective. Nevertheless, we suggest that the Hp1-1 phenotype may be a valuable genetic marker in assessing the possibility of various complications in people with diabetes mellitus.

Keywords

Hp1-1 · Hp2-1 · Hp2-2 · Hemoglobin · Genetic marker · Diabetes · Cardiovascular complications

Abbreviations

APOEA1	Apolipoprotein A1
APOEE4	Apolipoprotein E4
CAD	Coronary Artery Disease
CD	Crohn's Disease
CD163	Scavenger receptor 163
CD4	Scavenger receptor 4
CVD	Cardiovascular Disease
EGF	Epidermal Growth Factor
EGF-Hb	Complex of Epidermal Growth Factor with Haemoglobin
EGFR	Epidermal Growth Factor Receptor
Glu	Glutamine
Hb	Hemoglobin
HbA1c	Glycated hemoglobin
HDL	High-Density Lipoprotein
HIV-1	Human Immunodeficiency Virus type 1
HO-1	Heme Oxygenase 1
Hp	Haptoglobin
Hp1-1	Haptoglobin phenotype 1-1
Hp2-1	Haptoglobin phenotype 2-1
Hp2-2	Haptoglobin phenotype 22-2
Hp-Hb	Complex of haptoglobin with hemoglobin
IL-10	Interleukin 10
IL-6	Interleukin 6
IRSD	Iron-Regulated Surface Determinants
LDL	Low-Density Lipoprotein

Lys	Lysine
M1	Proinflammatory macrophage type M1
M2	Anti-inflammatory macrophage type M2
MMP/ADAMS	Matrix Metalloproteinases/A Disintegrin And Metalloproteinase
MS	Metabolic Syndrome
NO	Nitric Oxide
OS	Oxidative Stress
PAR2	Protease-Activated Receptor 2
preHp1	Prehaptoglobin 1
preHp2	Prehaptoglobin 2 (zonulin)
PSC	Primary Sclerosing Cholangitis
RA	Rheumatoid Arthritis
sCD163	Soluble form of CD163
Th1	Lymphocytes 1
Th2	Lymphocytes 2
TJ	Tight Junctions
TNFα	Tumor Necrosis Factor α
UC	Ulcerative Colitis

Key Facts of Haptoglobin Phenotype

1. Haptoglobin is an acute phase protein that is responsible for the uptake and removal of free hemoglobin in the blood by forming a complex with hemoglobin (Hp-Hb).
2. Two main haptoglobin polymorphs have been identified: Hp1 and Hp2, and three phenotypes: Hp1-1, Hp2-1, Hp2-2.
3. Haptoglobin is made up of four chains: two a and two p.
4. The structure of the chains determines the biological properties of the haptoglobin phenotypes.
5. Long chains of Hp2-2 with difficulty form a complex with Hb, which leads to an increase in oxidative stress and inflammation, and consequently contributes to the possible increase in the development of complications in diabetic patients.
6. Hp1-1 has protective properties because, as a low-mass molecule, it easily complexes with hemoglobin, activates the CD163 scavenging receptor and enhances the production of anti-inflammatory cytokines, including IL-10, which may limit the development of inflammatory diseases in the population.
7. Therefore, Hp1-1 can be considered an important and independent marker of diseases such as diabetes and its complications, e.g., cardiovascular diseases.

Introduction

Current epidemiological studies indicate that approximately 400 million people worldwide are affected by type 2 diabetes, and it is forecast that by 2035, this number will increase to over 700 million (Khan et al. 2020). Diabetes causes chronic

inflammation, which in turn leads to multiorgan complications that are a consequence of dysfunctional microcirculation and macroangiopathy. Glycemic disturbances cause changes in the functionality of the vascular mucosa and are the primary reason for the accelerated development of cardiovascular diseases, including atherosclerosis. It has been observed that in over 70% of cases, mortality in people with type 2 diabetes is associated with accelerated atherosclerotic changes (Moreno and Fuster 2004).

Over many years of research, the haptoglobin (Hp) genotype has been elucidated to play a pivotal role in cardiovascular risk. However, whether the Hp genotype is a significant marker in people with type 2 diabetes is unknown.

Structure and Polymorphic Variants of Haptoglobin

In the1940s, a protein called haptoglobin was discovered and described for the first time. Haptoglobin is an acute phase protein responsible for the uptake of free hemoglobin (Hb) in the plasma in the form of a haptoglobin-hemoglobin complex (Hp-Hb). This haptoglobin-hemoglobin complex is not filtered, preventing the loss of valuable iron in the urine and consequently preventing kidney damage. On the other hand, Hp-Hb, with the participation of macrophages, is removed by the reticuloendothelial system (primarily by the spleen) and thus inhibits the harmful oxidative activity of free hem by detoxifying reactive oxygen and nitrogen species.

Structurally, haptoglobin consists of two alpha chains (α1, α2) and two beta chains (β), covalently linked by a disulfide bond. Two basic haptoglobin polymorphs have been identified and described, designated Hp1 and Hp2, with three primary phenotypes: Hp1-1, Hp2-1, and Hp2-2. Homozygous individuals have type 1 alleles encoding Hp and are characterized by high expression of Hp1-1. The Hp2-2 phenotype occurs in homozygous people who have only type 2 alleles. Heterozygous people have both type 1 and 2 alleles, which are associated with high expression of Hp2-1.

In humans, the haptoglobin gene is located on the l6q22 chromosome that the first allele of the coding gene locus consists of 5 exons and 7 exons for allele 2. The Hp1 allele builds chains in two variants, differing by one amino acid. The Hp1F chain contains glutamine (Glu), while lysine (Lys) is present on the Hp1S chain. The Hp2 allele is presumed to exist only in humans and is formed by the duplication of intragenic exons 3 and 4 through the exchange of genetic material between non-homologous DNA crossing-over chromosomes of the Hp1F and Hp1S alleles. Therefore, the products of the Hp2 allele are longer chains with 388 amino acids as opposed to Hp1 (329 amino acids). In fact, in humans, the Hp1 and Hp2 alleles give rise to Hp1-1 dimers consisting of two αβ chains, Hp2-1 heterooligomers and Hp2-2 oligomers, which form covalent trimers and oligomers with more extensive αβ chains (Fig. 1) (Polticelli et al. 2008).

In humans, hepatocytes are primarily responsible for the production of haptoglobin, but the skin, lungs, kidneys, and, more recently, adipocytes have also been

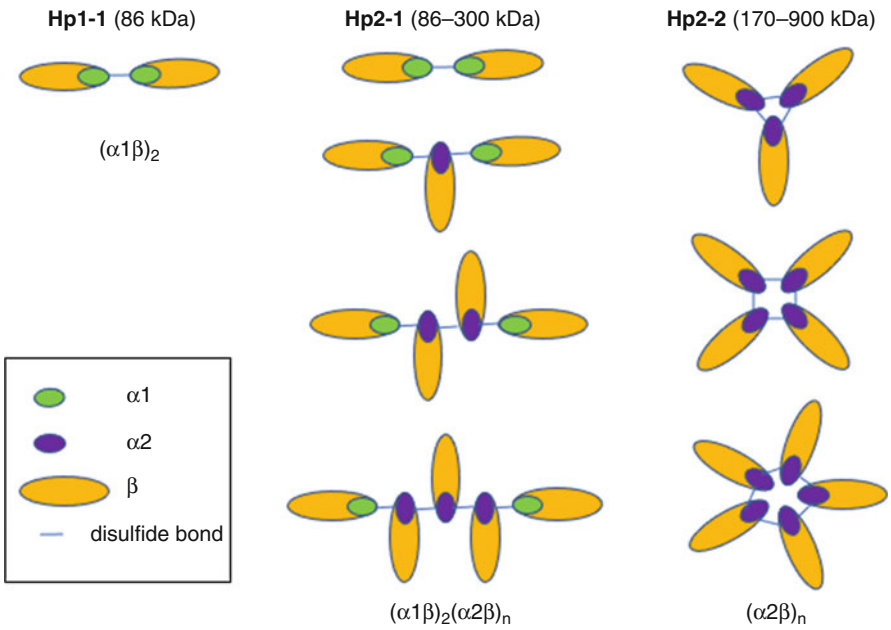

Fig. 1 Structures of haptoglobin phenotypes. This figure shows possible structural variants for the three haptoglobin phenotypes: Hp1-1, Hp2-1, and Hp2-2. (Source: own materials)

shown to produce this protein. Moreover, Hp expression has been detected in adipose tissue in other species, i.e.: rats and cattle.

The presence of Hb in human plasma is a physiological condition associated with intravascular hemolysis dependent on the breakdown of senescent erythrocytes. However, the condition becomes pathological in inflammatory diseases, autoimmune diseases, infectious diseases, such as malaria, dengue, or hemorrhagic fever, and in hereditary diseases, such as sickle cell anemia. The normal range of haptoglobin in the plasma is from 0.3–2.0 g/L. Abnormal concentrations are quite difficult to interpret. In inflammation or tissue damage, the Hp concentration increases many times above the normal range within 2 days, returning to normal after approximately 1 week. On the other hand, in acute hemolysis, Hp levels drop sharply and may return to normal within a week. This is due to large amounts of hemoglobin being released by lysis from erythrocytes that form complexes with haptoglobin (Shih et al. 2014).

Biological Functions of Haptoglobin

The structure of haptoglobin phenotypes determines its biological functions and properties. The short homodimeric chains of haptoglobin render Hp1-1 with a low molecular weight (86 kDa), which in turn facilitates the formation of complexes with

Hp-Hb. In contrast, the extensive multimeric chains forming Hp2-2 (170–900 kDa) and Hp2-1 (86–300 kDa) significantly increase its molecular weight, making it difficult to interact with hemoglobin and difficult to form the Hp-Hb complex. As a result, haptoglobin 2-2 is less protective and significantly increases the risk of cardiovascular complications, such as myocardial infarction, stroke, and kidney disease. It therefore seems that Hp may be a valuable genetic marker, especially in people with type 2 diabetes (Filipek et al. 2015; Stempkowska et al. 2020).

Note that although haptoglobin is present in all mammals, its polymorphic variants have only been identified in humans. The previously mentioned Hp1 and Hp2 variants are found primarily in European and American populations. It is known that the Hp2-1 variant (48%) is the most popular in the Western European population, and the Hp2-2 phenotype occurs in approximately 36% of the population. However, Hp1-1 is present in only 16% of the population. A different distribution of haptoglobin variants has been confirmed in Asia. Almost 90% of East and South Asian populations are characterized by the presence of Hp2-2 (Wobeto et al. 2008). Less popular variants of haptoglobin have also been discovered in these parts of the world. A haptoglobin polymorphism called HpDel has been identified in East and West Asian populations (Mongolia, China, Korea, Japan). The HpDel phenotype contains the Hp gene deletion allele minus a mass of 28 kb. Studies have confirmed that HpDel homozygotes do not produce haptoglobin and are phenotypically non-haptoglobinemic. In turn, HpDel heterozygotes are characterized by a decreased level of haptoglobin compared to people who have one of the basic Hp variants. Moreover, in people with HpDel (especially homozygotes), anaphylactic shock may occur if a blood transfusion is needed (Soejima et al. 2015).

It is probable that the deletion removing the Hp genes resulted in the Hp0 phenotype, which was detected in a Brazilian population. It has been suggested that a higher incidence of haptoglobin loss (HP0) is observed in patients with leukemia (Campregher et al. 2004). Another form of haptoglobin, Hp3-Johnson, which is a triple tandem repeat of the same stretch of DNA in a duplicate of the Hp2 gene, was detected for the first time in Japan. The Hp-Johnson variant consists α1 chain or α2 chains and an α3 chain, which has three times higher molecular weight than α1 chain. Hence, heterozygous Hp-Johnson is also written as Hp1-Johnson and Hp2-Johnson (Okazaki et al. 1998).

Correlations of Haptoglobin

Approximately 10% of free hemoglobin in humans is eliminated as the Hp-Hb complex, and 90% is eliminated by erythrophagocytosis. The CD163 macrophage scavenger receptor is actively involved in binding the Hp-Hb complex.

In humans, the CD163 (130 kDa) membrane protein is composed of a large extracellular region consisting of nine cysteine-rich class B domains, one transmembrane member and a C-terminus of varying length. There are four isoforms of the CD163 receptor. Three of them are well understood and encode CD163 proteins with different C-termini. The receptor form with the shortest terminal portion most

intensively mediates Hp-Hb endocytosis due to having the highest surface expression. In contrast, the other two isoforms of the CD163 receptor are involved in signal transduction during the intracellular response (Nielsen et al. 2006) (Fig. 2). Expression of the CD163 receptor is likely restricted to mature M2 macrophages, since CD163 activity has only been demonstrated on anti-inflammatory macrophages that appear during chronic inflammation, in the acute phase of infection, and during wound healing. The Hp-Hb complex links with CD163 through a high affinity bond that requires the presence of calcium ions (Ca^{2+}). CD163 has a greater affinity for Hp-Hb complexes containing a multimeric chain with the Hp1F phenotype than for complexes of hemoglobin and dimeric haptoglobin with the Hp1S phenotype. Therefore, CD163 readily binds complexes with Hp-Hb in which haptoglobin of the 1-1 phenotype is present. In contrast, in the case of Hp2-2, the CD163 receptor binds with difficulty to this Hp-Hb complex, which in turn results in an exacerbation of inflammation that is associated with endovascular hemolysis products.

During the process of endocytosis of the Hp-Hb complex, the hem subunit of hemoglobin is broken down by the enzyme hem oxygenase (HO). Of the 3 isoforms of HO, only hem oxidase 1 (HO-1) has an antioxidant effect throughout the human body by stimulating the degradation of free hem to carbon monoxide (CO), iron ions (Fe), and biliverdin, which is immediately degraded to bilirubin. As a result, HO-1 lowers the concentration of iron ions and induces the synthesis of ferritin. This process eliminates the generation of hydroxyl radicals, which are formed in the Fenton reaction from hydrogen peroxide with Fe^{2+}. Although there are many conflicting scientific reports, it is

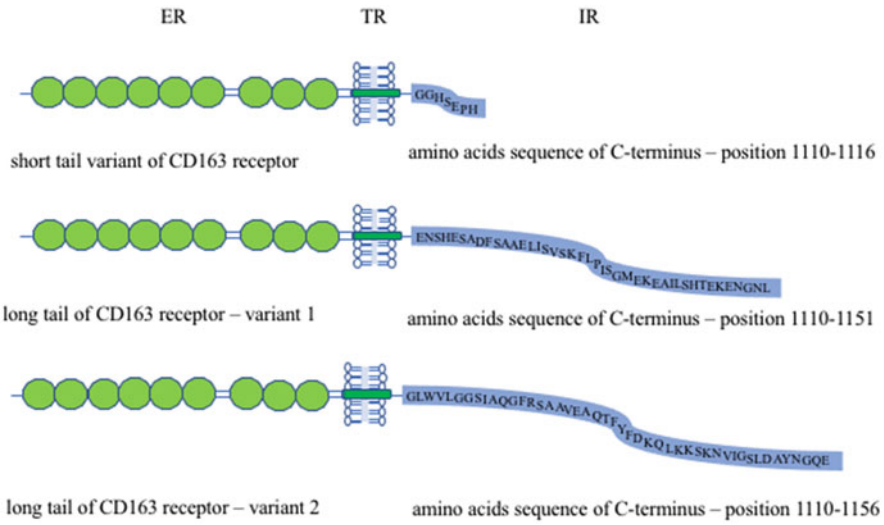

Fig. 2 The variants of CD163 receptor. ER – extracellular region, TR – transmembrane region, IR – intracellular region. This figure shows possible variants of the CD163 receptor. Each variant of the CD163 receptor is made up of an extracellular region (scavenger receptor class B domain), a transmembrane region, and a different length of an intracellular region (C-terminus tail). (Source: own materials)

believed that HO-1 has potent anti-inflammatory and cytoprotective properties (normal kidney cells, mucous membranes, lungs). Moreover, HO-1 protects tissues from oxidative damage and allows iron to be reused to build new hemoglobin particles.

Enhanced CD163 expression is induced by proinflammatory cytokines, such as interleukin 1 (IL-1) and interleukin 6 (IL-6). In contrast, interleukin 4 (IL-4), transforming necrotic factor α (TNF-α), and interferon-γ (IFN-γ) inhibit the activity of this receptor. However, recent studies have confirmed that the expression of CD163 and the possibility of forming complexes with Hb and various Hp phenotypes are also dependent on other anti-inflammatory mediators, e.g., interleukin 10 (IL-10) or transforming growth factor beta 1 (TGF-β). IL-10 is a 37 kDa homodimeric protein. In humans, the IL-10 receptor consists of two IL-10-1R chains and two IL-10-2R chains. Each of them contains 178 amino acids in various configurations. IL-10 activity is autoregulated through a negative feedback loop dependent on autocrine stimulation of the IL-10 receptor (Mosser and Zhang 2008). Moreover, it has been shown that high expression of the IL-10 receptor stimulates the activity of CD163 and the secretion of intracellular HO-1. The close correlation between CD163, IL-10, and HO-1 is effective in preventing not only oxidative tissue damage caused by free hemoglobin but also in attenuating the severity of inflammation.

As previously mentioned, CD163 is a macrophage membrane receptor. Natural factors (e.g., LPS, inflammatory mediators) facilitate detachment of the extracellular region, resulting in the appearance of a soluble form of CD163 (sCD163) in the plasma. The extracellular fragment is cleaved near the transmembrane segment of the membrane form of CD163 by recombination of SRCR domains 1-9. Many scientists are inclined to hypothesize that the concentration of sCD163 in the blood corresponds to the total pool of CD163 expression. It has been suggested that sCD163 activity is associated with inflammation, and sCD163 secretion depends on the anti-inflammatory and alternative activity of macrophages becoming the M2 phenotype. On the other hand, there are many scientific studies confirming the lack of a relationship between the membrane form of CD163 and its soluble form (sCD163).

In humans, under physiological conditions, sCD163 occurs in quite high concentrations, averaging 1.9 mg/L. In pathological conditions, levels of sCD163 secretion are significantly increased in patients with sepsis, pneumonia, myeloid leukemia and other diseases associated with increased monocyte proliferation. Increased levels of sCD163 were also observed in patients with rheumatoid arthritis (RA), but a higher concentration of the soluble form of the receptor was measured in the synovial fluid than in the serum. It was also confirmed that there is no correlation between sCD163 and CRP concentrations in RA patients, suggesting that sCD163 may not directly reflect the acute inflammatory phase. In 2006, an attempt was made to determine whether sCD163 is a marker of atherosclerosis. Approximately 150 patients were examined, primarily men aged 63 $\pm$ 11 years. The results revealed a significant increase in concentrations of sCD163 in the plasma of patients with advanced coronary disease. In this study, the concentration of sCD163 was correlated with the concentration of CRP and was independent of the age and sex of the patients. In contrast, in 2009, results were published that were completely different. The most common cause of ACS is the rupture of unstable plaques, which in turn causes

microhemorrhages. The released hemoglobin acts a strong proinflammatory stimulus due to the formation of reactive oxygen species (ROS). Free hemoglobin activates the entire cascade of metabolism, including an increase in CD163 expression on macrophages. However, whether the concentration of sCD163 is also increasing is uncertain. In a 2009 study, no statistically significant differences were found in different groups of patients with ACS (those with no cardiac changes, those with ACS but not STEMI, and those with ACS and STEMI). The authors speculated that factors such as Toll-like receptors and ADAMs metalloproteinase – present in coronary artery disease – inactivate the CD163 receptor, which in turn leads to the loss of cross-linking ability and the lack of a soluble form in the serum. Moreover, the CD163 receptor also loses its ability to bind to Hp-Hb complexes. Studies in groups of patients with type 2 diabetes also did not reach a definite answer regarding sCD163. In 2020, contrary to previous studies (2007), a close correlation was demonstrated between CD163 expression and the concentration of sCD163 in the plasma. In patients with Hp1-1, significantly higher levels of sCD163 were observed compared to patients with Hp2-1 and Hp2-2. Nevertheless, low concentrations of sCD163 in people with Hp2-1 and 2-2 may result from both an increase in CD163 expression (no correlation) and/or a decrease in CD163 synthesis. In people with type 2 diabetes, the Hp2-2 phenotype is closely correlated with the exacerbation of oxidative stress and inflammatory processes, which may result in increased expression of the CD163 receptor. However, it should be remembered that the protease receptors present in chronic pathological conditions may inhibit the release of the soluble form of CD163 from the surface of mature macrophages (Stempkowska et al. 2020; Levy et al. 2007).

An important role of haptoglobin is the modulation of lymphocytes in the immune system. Helper lymphocytes (Th) exist in two major subpopulations, Th1 and Th2. The primary role of Th1 lymphocytes is their participation in cellular responses, including in neoplastic and allergic diseases, viral infections, and protozoal diseases. Typical mediators produced by Th1 cells include interleukin 2 (IL-2), IFN-γ and TNF-α, which are associated with cytotoxic and inflammatory activities. Th2 lymphocytes play a key role in humoral responses, producing, among other things, IL-4 and IL-10. The nature of the Th1/Th2 equilibrium shift in favor of one subpopulation is characteristic of pathological states.

In 2003, in cellular and animal models, the relationship between the genotype of haptoglobin and the production of cytokines by lymphocytes was demonstrated for the first time. Hp1-1 and Hp2-1 have been shown to potentiate the production of Il-6 and IL10 to a much greater extent than Hp2-2. This activates Th2 and promotes a humoral response (Arredouani et al. 2003). In another study (2007), it was confirmed that the Hp1-Hb complex stimulates the secretion of IL-6 and IL-10 in contrast to the Hp2-Hb complex. Moreover, the release of these cytokines has been shown to be dependent on the CD163 receptor and the activity of casein kinase II. The Hp1 genotype modulates the balance of inflammatory (Th1) and anti-inflammatory (Th2) cytokines toward Th2 in macrophages stimulated by free hemoglobin (Guetta et al. 2007). These findings allow for a better understanding of the individual differences in an organism's response to microhemorrhage. They also suggest that people with Hp1-1 and 2-1 are better protected against infections.

The Relationship Between the Haptoglobin Variant and Cardiovascular Disease

There is a strong link between inflammation and oxidative stress (OS). The development of many inflammatory diseases (diabetes, atherosclerosis, cancer) is promoted by excessive oxidative activity. In the absence or under limited cleansing mechanisms, free Hb catalyzes the formation of free radicals, which in turn promote the oxidation of LDL cholesterol. The oxidized fraction of LDL is absorbed by macrophages and consequently forms lipid deposits in the walls of the arteries. Fatty deposits (atherosclerotic plaques) clog the blood vessels that supply blood to the heart and brain, increasing the risk of a heart attack or stroke. Studies have confirmed that in the case of Hp2-2 homozygotes, increased oxidative stress, dependent on free hemoglobin, significantly increases the risk of atherosclerotic plaque rupture and vascular thrombosis (Kalet-Litman et al. 2010).

In turn, the high-density lipoprotein (HDL) fraction is involved in reverse cholesterol transport and is responsible for cholesterol removal from macrophages. Due to its anti-atherosclerotic properties, HDL is recognized as a strong predictor of cardiovascular events. However, in type 2 diabetes, HDL often changes its properties, exacerbating inflammation and atherosclerotic changes. Moreover, deepening HDL dysfunction leads to an increase in cholesterol deposits in the artery walls and in early atherosclerosis. We can find an explanation for this state in several studies. Hemoglobin is an HDL protein and binds to apolipoprotein A-1 (APOEA1). Due to its structure and functions, the Hp genotype determines the properties of HDL. Allele 1 (Hp1-1) is reduced and forms a strong bond with Hb. As a result, HDL increases the outflow of cholesterol from macrophages. In contrast to Hp1-1, allele 2 (Hp2-2) binds poorly to Hb and increases the oxidation of HDL by Hb, resulting in a decreased ability to remove cholesterol from macrophages. Thus, we have strong evidence that the Hp2-2 phenotype increases HDL-associated Hb and correlates with the severity of vascular endothelial dysfunction in diabetic patients. On the other hand, it should be assumed that the Hp1-1 phenotype promotes proper APOEA1 signaling in endothelial cells, which increases repair functions, among others, by promoting the activation of nitric oxide (NO) synthase (Asleh et al. 2006, 2008, 2019; Watanabe et al. 2009).

The role of haptoglobin genotypes as an independent marker of cardiovascular disease (CVD) is still inconsistent. Several studies have shown no association between Hp polymorphisms and CVD. For example, Hp2-2 was found to be associated with the development of atherosclerotic plaques only in the carotid arteries but correlated with reduced blood triglycerides (Adams et al. 2013). Levy et al. (2004) found no correlation between Hp phenotype and the incidence of coronary heart disease. Moreover, people with diabetes and the haptoglobin Hp2-1 and Hp2-2 variants had a reduced number of coronary events. In a population study by Bruneck (a group of approximately 800 patients diagnosed with cardiovascular disease), Pechlaner et al. (2014) did not confirm a relationship between the haptoglobin phenotype and the risk of CVD in people with diabetes.

The hypothesis that there is no correlation between haptoglobin polymorphisms and the risk of cardiovascular disease has been refuted in many other studies. Acute myocardial infarction occurred 5 times more often in diabetic patients with Hp2-2 than in those without diabetes (Levy et al. 2002). This was likely related to several processes specific to diabetic conditions. Chronic hyperglycemia causes changes in the erythrocyte cell membrane, which leads to a reduction in erythrocyte viability. Additionally, it accelerates dysfunctional changes in the vascular endothelium. Glycated hemoglobin (HbA1c), which is elevated in people with diabetes, has been shown to be involved in the oxidation of both LDL and HDL. In addition, HbA1c enters into association with the Hp-Hb complex, which significantly enhances its oxidizing effect on lipoproteins and, consequently, leads to an increase in hemolysis, damage to the endothelium, and an increase in the concentration of glycated hemoglobin itself. The pro-oxidative properties of HbA1c are enhanced in the presence of Hp2-2 due to the impairment of the Hp2-2 protein to stabilize the hem in the glycated hemoglobin molecule. This relationship has been confirmed in several independent, long-term clinical trials. It was shown that patients with Hp2-2 and HbA1c $> 6.5\%$ were greater than 10 times more likely to develop coronary heart disease than Hp1-1 homozygotes with or without diabetes. Moreover, in Hp2-2 homozygotes with glycated hemoglobin concentrations below 6.5%, despite being diagnosed with diabetes, no increase in coronary events was observed (Cahill et al. 2013, 2015).

In turn, long-term (approximately 12 years) prospective studies in which over 300,000 people from the Swedish population participated proved that the Hp2-2 phenotype significantly increased the risk of acute myocardial infarction, stroke, and heart failure by 400%, 200%, and 150%, respectively. Moreover, a greater risk was observed in male than in female patients (Holme et al. 2009).

The potential protective properties of the Hp1-1 phenotype against the risk of heart disease in people with type 1 diabetes have been demonstrated in subsequent long-term prospective studies. After 18 years of research, it was found that the number of incidents of coronary artery disease (CAD) was decreased by fourfold in people with type 1 diabetes who had the Hp1-1 phenotype compared to people diagnosed with type 1 diabetes who had the Hp2-2 phenotype. In subsequent studies, as well as during 22 years of observation, there was a minimal trend toward lowering blood pressure and the incidence of stroke in people with Hp1-1 compared to Hp2-2 homozygotes. It should be noted that in the case of carotid embolism, much better protective effects of the Hp1-1 phenotype were demonstrated in silent brain infarcts (SBIs) than in lacunar strokes (Costacou et al. 2008, 2014, 2015; Staals et al. 2008).

The Relationship Between the Haptoglobin Variant and the Development of Diabetic Complications

In addition to cardiovascular disease, the primary complications associated with diabetes are retinopathy and nephropathy. Diabetic nephropathy comprises a structural and functional change in the kidney directly caused by hyperglycemia. The risk

of kidney damage is increased 12–17-fold in diabetic patients compared to healthy subjects. In a study of over 100 people with type 1 or type 2 diabetes and normal blood pressure values, none of the Hp1-1 homozygous patients developed symptoms of diabetic nephropathy. However, in those with Hp2-2 and Hp2-1, an increase in macroalbuminuria was observed in 35% and 27% of the examined people, respectively (Nakhoul et al. 2001). After 25 years of follow-up, Costacou T. and Orchard T.J. (2016) confirmed that the Hp2-2 variant increased cardiorenal mortality in patients with type 1 diabetes. However, in another study, no correlation was found between the haptoglobin variant and markers of diabetic nephropathy, i.e., microalbuminuria, macroalbuminuria and cystatin C. Therefore, scientists hypothesized that the protective properties of haptoglobin may be dependent on the age of the patient and the duration of diabetes mellitus. This was confirmed by showing that Hp1-1 protects against mortality in geriatric patients (>60 years of age) with chronic renal failure (Awadallah et al. 2008; Burbea et al. 2004a, b; Costacou et al. 2009).

A very common complication of diabetes is diabetic retinopathy, which occurs in more than half of patients with type 1 and type 2 diabetes. High blood glucose levels and frequently accompanying high blood pressure may increase blood flow. As a result of these processes, the membrane of the eyeball thickens, blocking the flow of fluids to and from the retina. In addition, the process of angiogenesis is intensified, the resulting blood vessels are easily damaged, and micro-bleeding occurs. Increasing inflammation can lead to visual impairment and even loss of vision. Data on the relationship between haptoglobin variants and the incidence of retinopathy are very limited. In a single study involving only 45 patients with type 2 diabetes and normal blood pressure, it was shown that retinopathy was more common in the Hp2-2 group (approximately 79% of patients) than in the Hp2-1 heterozygous group (approximately 44% of patients) (Mogarekar and Hampe 2013). On the other hand, Wu et al. (2017) performed a thorough meta-analysis of available research results from different regions of the world. Ultimately, it was not possible to confirm the hypothesis.

Relationship Between the Haptoglobin Variant and Neurodegenerative Diseases

Type 2 diabetes often results in cognitive decline, which increases with age and can lead to dementia (vascular dementia) and the development of neurodegenerative diseases, such as Parkinson's disease or Alzheimer's disease. Although the underlying mechanism of these diseases is not fully understood, it is believed that chronic hyperglycemia exacerbates oxidative stress, resulting in cerebrovascular disease and leading to microtrauma and neuronal death in the brain.

In the context of Parkinson's disease, it has been suggested that the Hp2 genotype is associated with a higher susceptibility to the idiopathic form of the disease (Costa-Mallen et al. 2015). In addition, in an animal model, a significant increase in the concentration of haptoglobin in the cerebrospinal fluid has been demonstrated in rats with induced neurodegenerative changes (Argüelles et al. 2010).

Cohort studies did not confirm a direct protective role of the Hp1-1 phenotype in Alzheimer's disease (Eikelenboom et al. 1984; Matsuyama et al. 1986). This is due to the pathological pathways of diabetes and Alzheimer's disease being different, as diabetes is primarily a peripheral disease, while Alzheimer's disease affects the central nervous system. However, it is known that the Hp1-1 variant effectively removes free hemoglobin and thus reduces chronic oxidative stress and inflammation in the body. It should therefore be assumed that in people with Hp1-1 and diabetes, neurodegenerative changes in the brain will progress much slower than in people with Hp2-2. Moreover, a common pathological mechanism between diabetes and Alzheimer's disease has been identified. One of the major risk factors is the deposition of beta-amyloid (βA) on the surface of nerve fibers, resulting in the appearance of protein aggregates and βA fibers in the brain. The βA proteins and the hyperphosphorylated tau proteins are neurotoxic. This process leads to the loss of nerve fibers and the appearance of cognitive issues in the elderly (Pereira et al. 2021). The E4 isoform of apolipoprotein E, which forms a complex with β, plays a special protective role. In fact, APOE4 removes βA and protects nerve fibers from its toxic effects. Spagnuolo et al. (2014) suggested that haptoglobin readily binds to APOE4 to displace βA, which in turn reduces the anti-neurodegenerative effect of APOE4. Unfortunately, data on the correlation between Hp and APOE are very limited, and it is not known what phenotype of Hp promotes complexes with APOE more. Based on the information described above, we can only speculate that Hp2-2 may have a greater affinity for APOE4 than Hp1-1. In particular, there are reports showing the lack of a protective role of the hippocampus against uncontrolled glycemia in elderly patients with Hp1-1 and type 2 diabetes (Livny et al. 2017).

Serious neurological consequences can also be the result of aneurysm rupturing in the abdominal aorta, thoracic system, or brain. The relationship between diabetes and aneurysms has not been well established. Moreover, many epidemiological studies have paradoxically reported a reduced risk of aneurysm rupture in people with type 1 or type 2 diabetes. This is related to the small diameter of the aorta in diabetic patients. The primary risk factors for aortic development and rupture are a large vessel diameter and a high rate of lesion growth (Radak et al. 2016). Nevertheless, it should be emphasized that in patients with subarachnoid hemorrhage, the Hp2 genotype increases the risk of complications of cerebral hypoxia with negative consequences (Blackburn et al. 2010).

The Relationship Between the Haptoglobin Variant and the Development of Infection

Another complication in diabetes is bacterial, fungal and viral infections. People with diabetes have reduced immunity and are more likely to develop the disease than healthy people. As mentioned earlier, the Hp2-2 phenotype increases susceptibility to infection, and the pathway is dependent on cells of the immune system, i.e., T lymphocytes, monocytes, macrophages, and dendritic cells. Dysfunction of the immune system is conducive to the development of many diseases, which, especially in people with

diabetes, may be life-threatening. Iron is necessary for the proliferation of bacteria from many species, including *Escherichia coli, Yersinia enterocolitica, Salmonella enterica serovar Typhimurium, Staphylococcus epidermidis, Staphylococcus aureus,* and *Corynebacterium diphtheriae* (Cross et al. 2015; Gianquinto et al. 2019). It can therefore be assumed that in the case of the Hp2 gene, hemoglobin unrelated to Hp supplies iron ions, which promotes the multiplication of many bacterial strains, including *Staphylococcus aureus*, which is responsible for the development of infections in soft tissues, the upper respiratory tract or the urinary tract (Pishchany et al. 2010; Dunyach-Remy et al. 2016). Iron recovery by *S. aureus* is mediated by receptor proteins called iron-regulated surface determinants (IRSD) and involves active hem extraction from Hb, transport, and release in bacterially infected plasma. Mikkelsen, Runager, and Andersen (2020) proposed Hp as a factor that directly inhibits receptor-mediated iron uptake by staphylococci. This mechanism is based on the formation of the Hp-Hb-IRSD complex, which means that the receptors lose their activity and are unable to extract hem from hemoglobin. Unfortunately, the authors of the study did not investigate the haptoglobin polymorphism. We can only assume that the properties described above have the Hp1-1 phenotype. However, this is not certain.

Despite these findings, the genetic variability of haptoglobin and its impact on infections is not conclusive. This is due to its pleiotropic nature, including immunomodulatory functions or participation in iron metabolism. In vitro studies have indicated the potential protective role of Hp2-2 and, to a lesser extent, Hp2-1 against *Streptococcus pyogenes.* The Hp2 gene promotes bacterial agglutination, and furthermore, this process is not correlated with iron uptake (Delanghe et al. 1998). In another study, Wasserzug et al. (2007) demonstrated that the Hp2 protein can more readily bind to the T antigen *Streptococcus aureus* and limit the multiplication of bacteria.

Returning to the fact that the structure of Hp2-2 does not allow the inhibition of oxidative stress or inflammation mediated by free hemoglobin, an animal model study reported a greater likelihood of developing acute respiratory syndrome in transgenic mice with a murine homologue of human HP2. Hp2-2 individuals were found to develop more severe acute pneumonia, damage to the lung endothelium, and mortality than Hp1-1 mice. In a prospective cohort study, the same team of researchers confirmed that Hp2-2 homozygotes are statistically more likely to develop sepsis than 1-1 homozygotes or 2-1 heterozygotes (Kerchberger et al. 2019).

With respect to other diseases, an association has been demonstrated between the Hp2-2 phenotype and increased mortality in tuberculosis and leprosy (Kasvosve et al. 2000; Seboka et al. 2017). However, in the case of human immunodeficiency virus type 1 (HIV), Hp2-2 homozygotes were shown to have a significantly reduced number of CD4-positive mononuclear cells. Therefore, the authors suggested a worse HIV-1 prognosis for Hp2-2 individuals. Interestingly, a protective role of HP0 was reported. Carriers of the Hp0 deletion gene had a significantly greater number of immune cells (CD4), which may be associated with a better prognosis in HIV-1 (Quaye et al. 2000). In contrast, Hp2-2 was associated with a reduction in the clinical symptoms of malaria, which may suggest a potential protective role for this genotype (Atkinson et al. 2007). Additionally, in the case of COVID-19, the Hp2 allele is assumed to play a favorable role due to its enhanced immune activity (Bandyopadhyay et al. 2021).

The Relationship Between the Haptoglobin Variant and the Development of Immune-Mediated Diseases

As mentioned earlier, an important role of haptoglobin is to regulate immune cells. In this context, an association has also been demonstrated between haptoglobin genotype and immune-related diseases. In a group of approximately 2000 patients with gastrointestinal diseases, i.e., ulcerative colitis (UC), Crohn's disease (CD), and primary sclerosing cholangitis (PSC), it was shown that there was a higher incidence of Hp2 in CD and UC patients than in controls. The same authors performed ex vivo studies to confirm the protective role of the Hp1 gene in experimental colitis. The pathway was dependent on Th1 and Th17 inhibition (Márquez et al. 2012). Paradoxically, the Hp1-1 phenotype has been identified as a potential risk factor in osteoarthritis of the knee and, to a lesser extent, in rheumatoid arthritis (RA) (Fernández-Costa et al. 2006). However, the results of studies from the 1980s clearly indicated a higher frequency of Hp2-2 in people with an acute course of RA (Dahlqvist and Fröhlander 1985).

Autoimmune diseases of the gastrointestinal tract cause inflammation of the intestines, which leads to an increase in their permeability and loss of the protective barrier between the host and external factors. More recently, zonulin has been identified as an inactive precursor to Hp2 and has been called prehaptoglobin 2 (preHp2). In fact, zonulin is a protein that modulates the permeability of the gastrointestinal wall, and its elevated concentration in the blood or feces is a marker of leaky gut. This phenomenon has been observed, in people suffering from diseases such as allergies, celiac disease, type 1 and 2 diabetes, obesity, rheumatoid arthritis, multiple sclerosis, or other autoimmune diseases, e.g., inflammatory bowel diseases. Research shows that zonulin levels correlate with glucose levels, blood dyslipidemia, inflammation, and insulin resistance (Fasano 2012).

Structurally, preHp1 and preHp2 are single-chain precursor proteins that, as a result of chain cuts (alpha and beta) and the formation of disulfide bridges, build mature forms of haptoglobin with appropriate phenotypes. Data on Hp precursor functions is very limited. There is some research into zonulin but not preHp1. In contrast to the split Hp2 double chain, single chain preHp2 destroys the intestinal barrier. The biological activity of the primary and mature forms results from different folding of the proteins in the chains. The pathological mechanism of preHp2 first involves activation of the epidermal growth factor receptor (EGFR) either directly through PAR2 or indirectly through the release of MMP/ADAMS. Moreover, the cellular enzyme tryptase IV cleaves zonulin into two subunits, eliminating one of the three disulfide bonds necessary to maintain EGF activity. Consequently, EGFR loses its ability to bind EGF. This molecule forms a complex with hemoglobin (EGF-Hb) and, intensifying inflammation, loosens the tight junctions (TJs) between intestinal epithelial cells (Fasano 2012; Vanuytsel et al. 2013; Zhang et al. 2017).

Environmental and genetic factors as well as cellular autoimmunity may correlate with metabolic syndrome (MS), which promotes the development of type 2 diabetes and atherosclerosis, significantly increasing the risk of cardiovascular complications. In the context of insulin resistance, it has been suggested that the Hp2 allele is

associated with a poorer response to glucose load, leading to increased blood glucose levels and reduced tissue sensitivity to insulin (Ricotti et al. 2020). Moreover, it was found that, in contrast to Hp1-1, the Hp2-2 phenotype strongly inhibited abdominal fat reduction and increased insulin resistance in obese women. The mechanism depends on the function of the Hp2 genotype and the production of proinflammatory cytokines (IL-6, TNF-α), which recruit proinflammatory monocytes/macrophages in white adipose tissue, causing chronic inflammation of adipocytes (Tanga et al. 2020).

Conclusion

In conclusion, recently, the haptoglobin genotype has been identified as an important and independent marker of diabetes, cardiovascular disease, and sickle cell disease. Hp2-2 contributes to an increase in complications because, due to its structure, it does not easily from Hp-Hb complexes, which in turn leads to an increase in oxidative stress and inflammation. Hp1-1, on the other hand, has protective properties because, as a low-mass molecule, it easily complexes with hemoglobin, activates the CD163 scavenging receptor and enhances the production of anti-inflammatory cytokines, including IL-10, which may limit the development of diseases in the population (Fig. 3). However, a review of the studies clearly shows that the relationship between Hp and the risk of complications or disease development is not linear. The Hp1-1 phenotype promotes repair functions, reduces inflammation, and protects LDL and HDL fractions from oxidation, ensuring their proper function. This biological activity is necessary in cardiovascular disease and appears to be one of the most common complications of type 2 diabetes. In addition, the Hp1-1 protein plays a key role in infectious diseases such as tuberculosis, influenza, HIV, and non-communicable diseases such as Alzheimer's disease, nephropathy, insulin resistance, and obesity and in bacterial infections, e.g., sepsis. In other diseases, such as malaria, rheumatoid arthritis, and COVID-19, the Hp1-1 phenotype plays an unclear role. Scientists suggest that the course of the disease is more severe in people with Hp1-1, as opposed to Hp2-2.

Can we modify the functions of haptoglobin by considering it a genetic marker? There seem to be such possibilities. There are vitamins (e.g., vitamin E) and compounds of natural origin (e.g., oleacein) that attenuate the pathological activity of Hp. Vitamin E is known to be a powerful antioxidant. It is therefore suggested that it reduces oxidative stress and inhibits the development of complications in people with type 2 diabetes who have the Hp2-2 genotype. In a cohort study of approximately 1500 patients, a significant reduction in coronary events, such as stroke and myocardial infarction, was observed after several months of vitamin E administration (Somer and Levy 2021). Other compounds with similar biological activities are polyphenols. The recently discovered oleacein is a secoidoid that is found in unfiltered extra virgin olive oil. Additionally, oleacein can be isolated from the leaves of Olea europea (Oleaceae) and Ligustrum vulgare (Oleacea). Oleacein has been shown to have powerful antioxidant and anti-inflammatory properties. In addition, oleacein can modify the functions of macrophages, changing their phenotype from M1

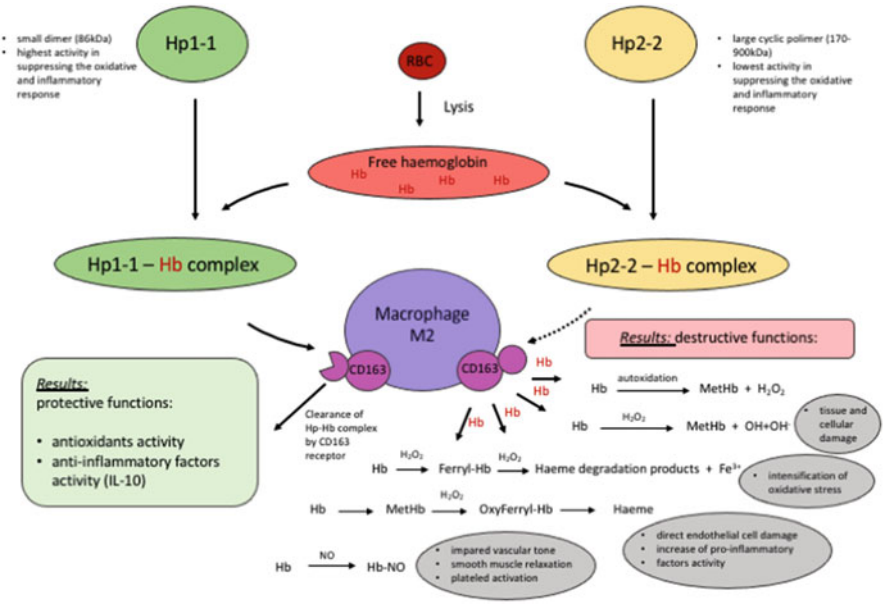

Fig. 3 Proposed scheme of biological activity of various haptoglobin phenotypes. In the case of Hp1-1: hemoglobin formed as a result of the breakdown of erythrocytes (ER) is bound in a complex with Hp1-1 and, with the participation of the CD136 scavenger receptor (only on mature anti-inflammatory macrophages M2), immediately removed from the body. As a result, we obtain a protective effect through increased antioxidant activity and anti-inflammatory (e.g., IL-10, HO-1). In the case of Hp2-2, the clearance is disturbed, which causes an increase in oxidative stress, which results in the activation of pro-inflammatory factors. It is known that chronic oxidative stress and inflammation lead to an increase in the possibility of developing complications, especially in people with type 2 diabetes, such as: retinopathy, nephropathy, bacterial and viral infections, ischemic heart disease, atherosclerosis, stroke or heart attack. This figure shows a diagram of the biological activity of two haptoglobin phenotypes: Hp1-1 and Hp2-2. (Source: Stempkowska et al. 2020)

(proinflammatory) to M2 (anti-inflammatory). Moreover, oleacein increases the activity of anti-inflammatory factors, i.e., the expression of Il-10, HO-1, and CD163, which modulates the functions of Hp2-2, leading to the formation of complexes with Hb and a reduction in oxidative stress (Filipek et al. 2015, 2017). Oleacein has also been shown to be effective in reducing the progression of early (reduction of foam cells) and late atherosclerotic (stabilization of atherosclerotic plaque) lesions, which is extremely important in people with diabetes (Filipek et al. 2015, 2017).

Summary Points

1. Haptoglobin is an acute phase protein that is responsible for the uptake and removal of free hemoglobin in the blood by forming a complex with hemoglobin (Hp-Hb).

2. Two main haptoglobin polymorphs have been identified: Hp1 and Hp2, and three phenotypes: Hp1-1, Hp2-1, Hp2-2.
3. Long chains of Hp2-2 with difficulty form a complex with Hb, which leads to an increase in oxidative stress and inflammation, and consequently contributes to the possible increase in the development of complications in diabetic patients.
4. Hp1-1 has protective properties because, as a low-mass molecule, it easily complexes with hemoglobin, activates the CD163 scavenging receptor and enhances the production of anti-inflammatory cytokines, including IL-10, which may limit the development of inflammatory diseases in the population.
5. The Hp1-1 phenotype promotes repair functions, reduces inflammation, protects LDL and HDL fractions from oxidation, thus ensuring their proper functioning.
6. The Hp1-1 protein plays a key role in cardiovascular disease, infectious diseases such as tuberculosis, influenza, and HIV, and non-communicable diseases such as Alzheimer's, nephropathy, insulin resistance, obesity, and in bacterial infections, e.g., sepsis.
7. Therefore, Hp1-1 can be considered an important and independent marker in people with type 2 diabetes.

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Part IV

Functional Variables, Physiological Variables, and Platforms

Day-by-Day Home Blood Pressure Monitoring as a Biomarker in Diabetes

34

Links with Functional Variables [eGFR and Albuminuria]

Daisuke Suzuki, Satoshi Hoshide, and Kazuomi Kario

Contents

Introduction 707
Diabetes and BPV 708
Different Types of BPV and Related Evidence 708
Day-by-Day HBPV and Target Organ Damage or CVD 710
Current Status of DKD 711
Association Between HBP, Day-by-Day HBPV, and DKD 713
Perspectives on HBPM 718
Conclusion 718
Applications to Prognosis and Other Diseases or Conditions 718
Applications to Prognosis 718
Applications to Other Diseases or Conditions 719
Mini-Dictionary of Terms (5–15 terms) 719
Key Facts 719
Summary Points 721
References 721

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Abstract

Home blood pressure (HBP) is an indispensable tool for hypertension management, because both increased mean HBP and day-by-day HBP variability (HBPV) have been associated with target organ damage and cardiovascular disease (CVD) risk independent of office blood pressure (OBP). Although hypertension is an important risk factor for diabetic kidney disease (DKD), data are sparse regarding the association between HBP or HBPV and DKD. The J-HOP study demonstrated that increased mean HBP levels were associated with albuminuria in both patients with and without diabetes, while increased day-by-day HBPV was associated with decreased renal function only in patients with diabetes. The burden of BP level and BPV may differ according to the phenotype of DKD, as classified by albuminuria and the estimated glomerular filtration rate (eGFR). HBP management using information and communication technology would provide a new strategy for risk stratification and prevention of CKD in the future.

Keywords

Albuminuria · CVD · Day-by-day HBPV · Diabetes · DKD · eGFR · HBP · ICT · OBP · Target organ damage

Abbreviations

ABPM	Ambulatory blood pressure monitoring
AOBP	Automated office blood pressure
ARV	Average real variability
baPWV	Brachial-ankle pulse wave velocity
BP	Blood pressure
BPV	Blood pressure variability
cfPWV	Carotid-femoral pulse wave velocity
COVID-19	Coronavirus disease 2019
CV	Coefficient of variation
CVD	Cardiovascular disease
DKD	Diabetic kidney disease
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
HBP	Home blood pressure
HBPM	Home blood pressure monitoring
HBPV	Home blood pressure variation
HF	Heart failure
HSBP	Home systolic blood pressure
ICT	Information and communication technology
NT-pro BNP	N-terminal pro brain-type natriuretic peptide
OBP	Office blood pressure
PWV	Pulse wave velocity
RAAS	Renin-angiotensin-aldosterone system

SBP	Systolic blood pressure
SD	Standard deviation
SHATS	Systemic hemodynamic atherothrombotic syndrome
UACR	Urine albumin-to-creatinine ratio
VIM	Variability independent of the mean

Introduction

Hypertension and diabetes are major causes of microvascular diseases (e.g., kidney disease, retinopathy) and macrovascular disease (e.g., stroke, heart failure (HF), and coronary artery disease) (Umemura et al. 2019; American Diabetes 2021a, b). A previous study demonstrated that diabetes and hypertension are independent risk factors for stroke and the risk is enhanced when both are present (Hu et al. 2005). Thus, in patients with diabetes, optimal blood pressure (BP) control is an important component of the management to prevent cardiovascular disease (CVD). However, the Action to Control Cardiovascular Risk in Diabetes Blood Pressure (ACCORD-BP) trial showed that lowering systolic blood pressure (SBP) to <120 mmHg did not reduce the composite outcome (nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death) compared with lowering SBP to <140 mmHg in diabetic patients (Accord Study Group et al. 2010). Another study on diabetic patients reported that the home blood pressure (HBP) level was associated with CVD events, but office blood pressure (OBP) was not (Ushigome et al. 2021). This discrepancy may be explained by the different methodologies of BP measurement. In clinical practice, as an out-of-office BP measurement, ambulatory blood pressure monitoring (ABPM) and HBP measurement are recommended for the diagnosis and management of hypertension (Umemura et al. 2019; Whelton et al. 2018; Williams et al. 2018; Kario et al. 2019; Hoshide et al. 2020). In real-world settings, HBP measurement is preferable, because ABPM is more invasive and less convenient for both physicians and patients despite a number of studies showing that ABPM is clinically relevant (Kario et al. 2019; Umemura et al. 2019). The Ohasama study was the first to show that HBP was more predictive of mortality than OBP in a large cohort from the general population (Ohkubo et al. 1998). The Home Blood Pressure Measurement with Olmesartan Naive Patients to Establish Standard Target Blood Pressure (HONEST) study, a large study that included more than 20,000 Japanese patients with essential hypertension who were newly administered olmesartan, demonstrated that morning HBP was a predictor of coronary artery disease and stroke (Kario et al. 2016). The Japan Morning Surge-Home Blood Pressure (J-HOP) study, a nationwide observational study that enrolled over 4,000 Japanese patients with at least 1 cardiovascular risk factor or a history of CVD, showed that morning HBP improved the discrimination of stroke beyond that achieved by OBP and other cardiovascular risk factors (Hoshide et al. 2016). Thus, HBP measurement has been associated with a risk of CVD independent of OBP, and its predictive ability is better than that of OBP (Ohkubo et al. 1998; Kario et al. 2016; Hoshide et al. 2016).

HBPM can be used to measure blood pressure variability (BPV), and the difference between morning and evening BP levels or maximum mean HBP has been associated with risk for stroke or other CVDs (Narita et al. 2021b; Fujiwara et al. 2021). HBPM can also detect seasonal BPV. This is important, because BP levels have been associated with temperature (Umishio et al. 2021; Kubozono et al. 2021), and there are differences in the BPV pattern between seasons (Narita et al. 2020, 2021a). In addition, HBPM is available to measure BP every day over the long term, thereby allowing the assessment of day-by-day BP variability. Increased day-by-day home blood pressure variation (HBPV) has been associated with both target organ damages and new onset of CVD independent of the mean HBP level (Ishiyama et al. 2020; Godai et al. 2020; Hoshide et al. 2018). However, to accurately evaluate multiple home BP readings and easily utilize them in clinical practice, an information and communication technology (ICT)-based system is essential.

Diabetic kidney disease (DKD) has been attracting attention, because the phenotype of DKD has changed due to aging and use of RAAS blockers (Hirakawa et al. 2017). The phenotypes of DKD are divided into DKD with albuminuria, lower eGFR, or both. Albuminuria is associated with new onset of CVD events (Schmieder et al. 2007). Lower eGFR is associated with increased mortality (Hallan et al. 2012). Hypertension is one of the most important risks for DKD. Although the prevention and improvement of albuminuria and lower eGFR are important, whether lower BP control prevents the progression of DKD has been debated. There is scant evidence that intensive BP treatment improves renal outcome and the target BP for DKD differs among the different guidelines (Whelton et al. 2018; Williams et al. 2018; Georgianos and Agarwal 2021). Thus, DKD and related CVD events may not be linked to mean BP level, but rather to other BP parameters. A recent paper reported that not mean BP but BPV was associated with lower eGFR in diabetes patients (Suzuki et al. 2020). BPV may thus be a more important BP parameter than mean BP in patients with DKD.

In this chapter, we discuss the clinical significance of BPV for diabetes, with a particular focus on DKD.

Diabetes and BPV

Different Types of BPV and Related Evidence

Because BP fluctuates over both the short and long term, BPV is classified into some categories: beat-to-beat, diurnal, day-by-day, visit-to-visit, seasonal, and yearly BPV (Fig. 1) (Kario 2015). In general, BPV is a temporal response to diverse environmental stimulations, including physical and mental stressors that allow the cardiovascular system to function properly (Cuspidi et al. 2021; Parati et al. 2020). However, a sustained increase in BPV may represent a change in cardiovascular mechanisms, which may itself lead to target organ damage and cardiovascular events (Parati et al. 2020). Currently, there are several indexes of BPV that are variously evaluated by OBP, HBP, or ABPM. The associations among these BPV indexes are

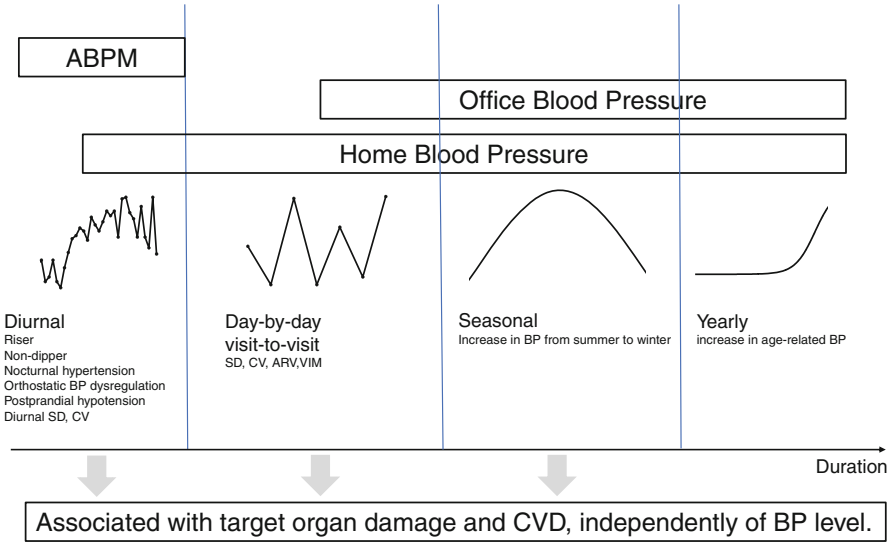

Fig. 1 Type of BPV. BPV includes diurnal, day-by-day, visit-to-visit, seasonal, and yearly. This variability could be detected by ABPM, OBP, and HBP. BPV is associated with target organ damage and CVD, independent of BP level

Table 1 Characteristics of BPV in patients with diabetes

Nocturnal hypertension
Non-dipper/riser pattern
Masked hypertension
Orthostatic BP dysregulation (orthostatic hypotension and hypertension)
Postprandial hypotension

not strong, suggesting that each BPV index may represent a different aspect of BPV (Abellan-Huerta et al. 2018). A previous study showed that each of these BPV indexes is associated with CVD and the prognostic power of BPV for CVD was higher than that of mean BP (Stevens et al. 2016). However, that study was unable to determine which BPV index was most effective for the prediction of CVD.

Previous studies reported that patients with diabetes were more likely to have unique BPV patterns, such as nocturnal hypertension, a non-dipper/riser pattern, orthostatic BP dysregulation (orthostatic hypotension and hypertension), or postprandial hypotension, compared to those without diabetes **(**Table 1**)** (de la Sierra et al. 2009; Cuspidi et al. 2017; Chang et al. 2018; Yoshinari et al. 2001; Thoonkuzhy and Rahman 2020). These BPV patterns in diabetic patients may be explained by autonomic neuropathy and progressed arterial stiffness (Eguchi et al. 2009); the same patterns have also been associated with a risk of CVD (Kario et al. 2020, 2021a; Hoshide et al. 2021, 2008, 2012; Fujiwara et al. 2020). For example, both riser and non-dipper patterns have been associated with target organ damage and CVD (Komori et al. 2017; Kario et al. 2020). The Japan Ambulatory Blood

Pressure Monitoring Prospective (JAMP) study, a nationwide multicenter observational study including patients with at least one CVD risk factor, demonstrated that every 20 mmHg increase in nighttime SBP led to an increase in the risks of CVD (hazard ratio 1.21; 95%CI 1.03–1.41) and HF (hazard ratio 1.36; 95%CI 1.08–1.71) and abnormal diurnal BPV (i.e., a riser pattern) was related to higher risks of CVD (hazard ratio 1.48; 95%CI 1.05–2.08) and HF (hazard ratio 2.45; 95%CI 1.34–4.48) compared with normal diurnal BPV (a dipper pattern) (Kario et al. 2020).

In diabetic patients, several studies have reported that higher BPV confers a risk for CVD (Cardoso et al. 2021; Kilpatrick et al. 2010; Hata et al. 2013; Wan et al. 2017; Kaze et al. 2021; Yu et al. 2019). In patients with type 2 diabetes, increased short-term BPV such as that measured by the standard deviation (SD) of ABPM, particularly daytime diastolic BPV, was a risk for new onset of CVD and mortality, but not for microvascular disease (neuropathy, retinopathy, and nephropathy) (Cardoso et al. 2021). In patients with type 1 diabetes, both the OBP level and visit-to-visit BPV assessed by office BP were independent predictors of the development of nephropathy (Kilpatrick et al. 2010). In patients with type 2 diabetes, visit-to-visit BPV was a risk factor for microvascular disease (retinopathy or new or progression nephropathy), CVD events, and mortality (Hata et al. 2013; Wan et al. 2017; Kaze et al. 2021; Yu et al. 2019). Thus, BPV may be an important biomarker of CVD in patients with diabetes.

Day-by-Day HBPV and Target Organ Damage or CVD

Day-by-day HBPV is one of the types of BPV assessed by HBP. Day-by-day HBPV can be evaluated using the SD, coefficient of variation (CV), average real variability (ARV), or variability independent of the mean (VIM) as a statistical method. As shown in Fig. 2, SD is the standard deviation of the BP measured on each day, and

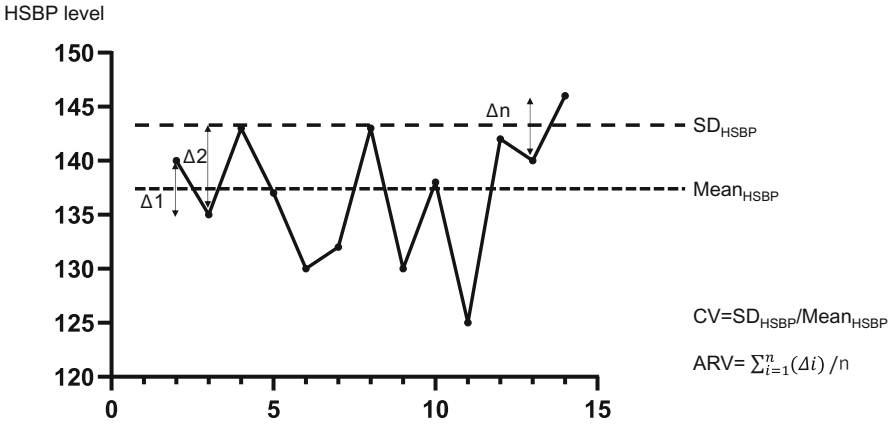

Fig. 2 Day-by-day HBPV index. SD is the standard deviation of the BP measured on each day, and CV is SD divided by the mean BP. ARV is estimated as the absolute difference between each of the BP values on consecutive days

CV is the SD divided by the mean BP. ARV is estimated as the absolute difference between each of the BP readings on consecutive days, and unlike when determining SD and CV, the order of BP measurements is also taken into account when determining ARV (Hoshide et al. 2018). SD, CV, and ARV are partially related to the BP level, and correcting for BP may not completely remove the influence of BP level. The VIM, which is an index of day-by-day HBPV unrelated to mean BP, was estimated by a fitting curve using the mean SBP and SD of SBP (Dolan and O'Brien 2010). Because VIM is not a clinical measurement but a statistical tool (Dolan and O'Brien 2010), it is not suitable for use by physicians in clinical practice.

Each of these day-by-day HBPV indices is considered to be a predictor of the risk of target organ damage and onset of CVD, but it is unclear which index is the best prognostic marker. The J-HOP study showed that the adjusted hazard ratios (95%CI) of a 1-SD increase in SD, CV, ARV, and VIM of day-by-day HBPV for the risk of CVD were 1.32 (95%CI 1.14–1.53), 1.32 (1.14–1.52), 1.25 (1.10–1.41), and 1.32 (1.14–1.52), respectively. Thus, there was no remarkable difference in the risk of CVD among day-by-day HBPM indexes (Hoshide et al. 2018).

Current Status of DKD

DKD is one of the risk factors for end-stage renal disease (ESRD) in patients with diabetes. Although the definition of DKD is still under discussion (Hirakawa et al. 2017), previous studies defined DKD as kidney disease accompanied with diabetes mellitus, regardless of renal biopsy (Hirakawa et al. 2017; Yokoyama et al. 2020). The DKD phenotypes include DKD with albuminuria, lower eGFR, or both (Fig. 3). Classically, diabetic nephropathy was initially characterized by the presence of albuminuria in patients with diabetes. Subsequently, the patient with albuminuria develops macroalbuminuria and experiences a decrease in eGFR, leading to ESRD (Nelson et al. 1996; Gaede et al. 1999). This pathway is based on the glomerular hypertension pathway. Glomerular hypertension, caused by various factors such as diabetes, leads to irreversible nephron damage and progression of DKD (Brenner et al. 1996; Tonneijck et al. 2017). However, some diabetic patients show a decrease in eGFR without the appearance of albuminuria.

RAAS blockers are innovative drugs for DKD. RAAS blockers reduce intraglomerular pressure and result in albumin excretion (Leoncini et al. 2020). Several studies have reported that the use of RAAS blockers reduces albuminuria in patients with diabetes (Viberti et al. 2002; Haneda et al. 2004). Epidemiologically, the prevalence of DKD without albuminuria has increased, and the prevalence of mortality in patients with DKD without albuminuria increased in 2007–2010 compared to that in 1988–1994 (Kramer et al. 2018). Pathologically, kidney tissue in patients with DKD with albuminuria undergoes changes such as mesangial extracellular matrix accumulation and glomerular basement membrane thickening (Gnudi et al. 2016), whereas kidney tissue in patients with DKD without albuminuria is characterized by progresses to vascular and tubulointerstitial lesions like those in hypertension-associated nephrosclerosis (Shimizu et al. 2014). DKD leads to ESRD

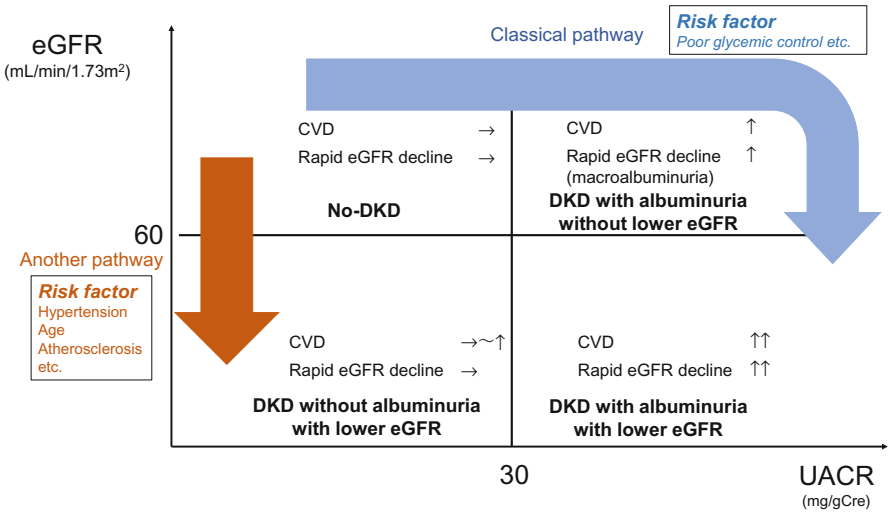

Fig. 3 Phenotypes of DKD and risk of CVD and rapid eGFR decline. DKD is classified into four phenotypes according to the presence or absence of albuminuria and reduced eGFR. Each of the four phenotypes may have a different risk of CVD and rapid eGFR decline. Classically, the decline in eGFR occurs after the appearance of albuminuria. However, there are types of eGFR decrease without albuminuria. Each pathway has risk factors

irrespective of the presence of albuminuria. The different phenotypes of DKD are characterized by different pathologies, and these pathologies in turn make different contributions to CVD occurrence and renal disease progression.

However, it remains a matter of debate whether DKD patients without albuminuria have worse prognosis compared to those with albuminuria. The Renal Insufficiency and Cardiovascular Events Italian Multicentre (RIACE) study reported that both patients with albuminuria but not lower eGFR and patients with lower eGFR but not albuminuria had higher CVD risk than those without either albuminuria or lower eGFR in an Italian population (adjusted hazard ratio, 1.45; 95%CI 1.33–1.58 and 1.58; 95%CI 1.43–1.75, respectively) (Penno et al. 2018). In a study enrolling Japanese patients with diabetes (the Japan Diabetes Clinical Data Management [JDDM] study), the patients with albuminuria but not lower eGFR had higher CVD risk than those without either albuminuria or lower eGFR (adjusted hazard ratio 1.75; 95%CI 1.32–2.3), whereas those with lower eGFR but not albuminuria did not have a higher risk of CVD (adjusted hazard ratio 1.06; 95%CI 0.63–1.79) (Yokoyama et al. 2020). This discrepancy may be explained by differences in the race or background of patients. Enrolled patients in the RIACE study were older and higher SBP levels than those in the JDDM. Age and hypertension are strongly linked to arteriosclerosis. In diabetic patients with advanced arteriosclerosis, DKD represented as lower eGFR may be a higher risk for CVD, regardless of the presence of albuminuria.

However, a previous study reported that the risk of eGFR decline in patients with lower eGFR but not albuminuria was lower than that in patients with albuminuria but

not lower eGFR (Yokoyama et al. 2020). Another study showed that diabetic patients with albuminuria and lower eGFR had a higher rate of eGFR decline than those with lower eGFR but not albuminuria (Koye et al. 2018). Those studies suggested that the presence of albuminuria would be a determinant of eGFR decline, i.e., DKD progression. Moreover, other reports found that 10% of type 1 diabetic patients without albuminuria experienced a rapid decline of eGFR [$\geq$3.3% per year (this value means 2.5th percentile of the distribution of renal function decline in the general population (Lindeman et al. 1985; Krolewski et al. 2014))] as calculated by the combination of serum creatinine and cystatin C and the risks for rapid decline of eGFR were lower level of baseline eGFR; higher age; higher levels of uric acid, SBP, and tumor necrosis factor receptor 1 or 2; and higher diabetes duration in patients with type 1 diabetes and non-albuminuria (Krolewski et al. 2014). Another study demonstrated that advanced arterial stiffness assessed by baPWV was also associated with lower eGFR in patients without albuminuria (Kume et al. 2019). A multicenter, open-label study demonstrated that intensive therapy for multifactorial factors such as high glucose, BP, and lipid levels reduced the incidence of renal outcome compared with a standard treatment group (hazard ratio 0.68; 95%CI 0.56–0.82) in diabetic patients (Ueki et al. 2017). Especially, intensive glucose control alone was not sufficient to realize an improvement in renal outcome compared to a standard treatment group (Ueki et al. 2017; Advance Collaborative Group et al. 2008; Perkovic et al. 2013). To prevent DKD progression, total cardiovascular risk management is necessary.

Association Between HBP, Day-by-Day HBPV, and DKD

The J-HOP study examined whether the mean home BP level and the day-by-day HBPV have different associations with albuminuria and lower eGFR in patients with and without diabetes (Suzuki et al. 2020). The results showed that, in both patient groups, increased mean home BP levels were associated with albuminuria, while increased day-by-day HBPV was associated with lower eGFR only in the patients with diabetes (Fig. 4 and Table 2) (Suzuki et al. 2020). Depending on the phenotype of DKD classified by albuminuria and eGFR, there may be differences in the burden between the BP level and BPV (Suzuki et al. 2020).

Association Between HBP Level and Albuminuria

Hypertension is one of the most important risk factors for albuminuria. Increased OBP has also been associated with albuminuria and progression of albuminuria (Schmitz et al. 1994). Mean HSBP levels have also been associated with albuminuria, and this association has been observed independent of the OBP level (Suzuki et al. 2020). This association has also been confirmed in other populations (Matsumoto et al. 2017; Gaborieau et al. 2008). In patients with uncontrolled morning HBP, the titration of bedtime dosing of an alpha-adrenergic blocker significantly reduced morning HBP and albuminuria, and the reduction in morning HBP was associated with decreased albuminuria (Kario et al. 2008). Moreover, the

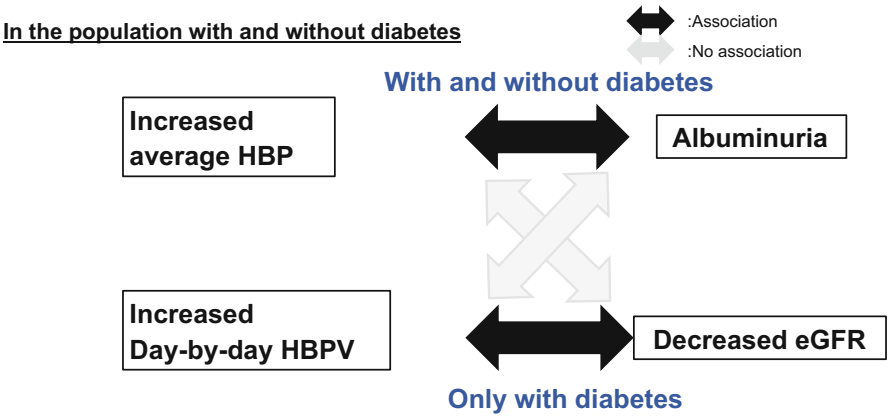

Fig. 4 Association between increased average HBP or increased day-by-day HBPV and albuminuria or decreased eGFR. Increased HBP was associated with albuminuria. On the other hand, increased day-by-day HBPV was associated with decreased eGFR only in patients with diabetes

administration of a RAAS blocker at bedtime reduced morning HBP and albuminuria, but there was no significant difference in the reduction of morning HBP between the administration of a RAAS blocker at bedtime and in the morning (Kario et al. 2010). Interestingly, however, the reduction in albuminuria was greater by the administration of a RAAS blocker at bedtime than by morning administration (Kario et al. 2010).

Association Between Day-by-Day Home BPV and Albuminuria

The J-HOP study demonstrated that day-by-day HSBP was not associated with albuminuria in patients with diabetes (Suzuki et al. 2020). On the other hand, the KAMOGAWA study, which enrolled 858 patients with type 2 diabetes, revealed that day-by-day HSBP as evaluated by CV was associated with albuminuria independent of CVD risks, including mean HBP level (Ushigome et al. 2011). The KAMOGAWA study also reported that day-by-day morning home SBP was a predictor for the progression of albuminuria, but day-by-day home evening SBP was not (Ushigome et al. 2018). This discrepancy may be explained by differences in the study populations. Namely, the rate of antihypertensive medication use and the mean HBP in the J-HOP study were higher than those in the KAMOGAWA study. This suggests day-by-day HBPV may have an impact on albuminuria in populations with relatively good HBP control. However, further research will be needed to determine whether HBPV is useful as a predictor of progression of albuminuria.

Association Between HBP Level and eGFR

The J-HOP study also showed that the mean HSBP level was not associated with lower eGFR, independent of CVD risks including OBP in patients with diabetes (Suzuki et al. 2020). Other previous studies also found that there was no association between the OBP level and eGFR in other populations. In a study on 150 patients

Table 2 Multiple linear regression analysis of SBP variables for log-transformed UACR and eGFR. The models of average BP included age, sex, BMI, hyperlipidemia, prevalent cardiovascular disease, antihypertensive drug, and office SBP. The models of CV and ARV included the covariates used in the model of average BP and the average morning or evening SBP. The β-value expresses the risk associated with a 1-SD increase in the explanatory variables (Suzuki et al. 2020)

	Log-transformed UACR					eGFR				
	Without diabetes (n = 3197)		With diabetes (n = 1034)			Without diabetes (n = 3197)		With diabetes (n = 1034)		
BP variables	β	p	β	p	p for interaction	β	p	β	p	p for interaction
Morning SBP										
Average BP, per 1SD	0.211	<0.001	0.276	<0.001	0.062	0.189	0.560	0.742	0.254	0.828
CV, per 1SD	0.024	0.225	0.018	0.674	0.526	−0.432	0.119	−1.373	0.011	0.103
ARV, per 1SD	0.037	0.069	0.112	0.014	0.097	−0.338	0.245	−1.650	0.003	0.055
Evening SBP										
Average BP, per 1SD	0.172	<0.001	0.257	<0.001	0.036	−0.848	0.006	−0.837	0.184	0.872
CV, per 1SD	0.003	0.861	−0.017	0.689	0.416	−0.042	0.881	−1.169	0.030	0.029
ARV, per 1SD	0.019	0.361	−0.018	0.686	0.588	−0.084	0.771	−1.206	0.030	0.043

with CKD patients, OBP was not associated with lower eGFR (Nakano et al. 2015). In a meta-analysis, a reduction in OBP did not reduce the risk of renal outcome defined by ESRD, transplantation, or death (Ettehad et al. 2016). Not only higher SBP but also lower SBP was associated with faster declines of eGFR, i.e., the J-curve phenomenon (Jafar et al. 2003). Renal failure is caused by multiple pathological mechanisms, and both lower and higher BP may contribute to its formation (Ettehad et al. 2016).

Association Between Day-by-Day Home BPV and eGFR

In the J-HOP study, the association between an increase in day-by-day home BPV and lower eGFR was confirmed in patients with diabetes, but not in those without (Suzuki et al. 2020). In a cross-sectional study of 268 patients with type 2 diabetes, increased day-by-day HSBP was also associated with lower eGFR (Nishimura et al. 2013). However, in a study of 135 patients with stage 3–5 CKD, day-by-day HBPV was not related with progression of CKD (Okada et al. 2012). These previous studies suggest that day-by-day HBPV may be associated with lower eGFR only in patients with diabetes.

In order to explain the association between HBPV and renal function, we propose the concept of SHATS to describe the relationship between hemodynamic stress, including BPV, and vascular disease (Fig. 5) (Kario 2015, 2019).

While hemodynamic stress is composed of BPV and central/focal BP, vascular disease is composed of large artery disease and strain vessel disease. Exaggerated hemodynamic stress leads to a progression of vascular disease, and, in a vicious cycle, progression of vascular disease itself leads to an increase in hemodynamic stress. In addition, this cycle is further accelerated by risk factors such as diabetes, dyslipidemia, thrombotic risk, inflammation, and CKD (Kario 2013). In this way,

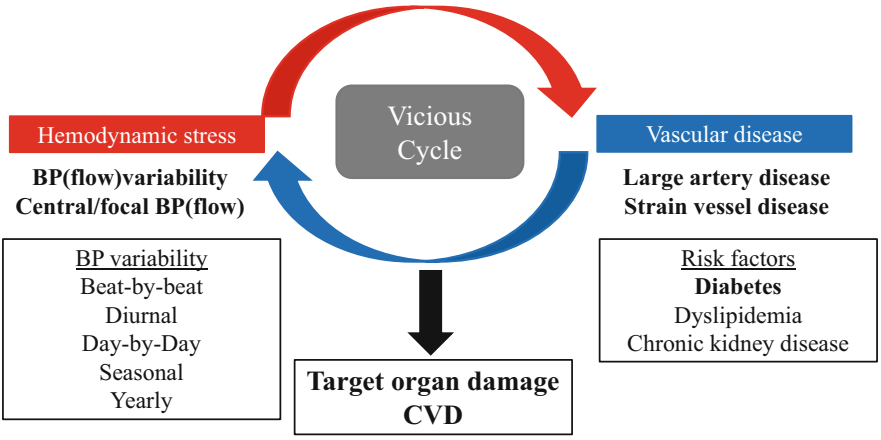

Fig. 5 The concept of systemic hemodynamic atherothrombotic syndrome. Hemodynamic stress such as BPV and vascular disease form a synergistic vicious circle and result in target organ damage and CVD events. Diabetes is one of the risk factors of SHATS

hemodynamic stress and vascular disease have a negative synergistic effect on each other, exacerbating their respective elements and resulting in target organ damage and cardiovascular events. Specifically, the J-HOP study also demonstrated that a group with higher baPWV (≥1800 cm/s), which is widely used as surrogate marker of arterial stiffness, exhibited a greater association between a higher day-by-day HBPV index and NT-pro BNP than did a group with lower baPWV (<1800 cm/s) and there was an interaction between day-by-day HBP variability and NT-pro BNP according to the presence of higher baBWV (Ishiyama et al. 2020). In addition, the day-by-day HBPV index was associated with new onset of CVD events only in the higher baPWV group (≥1800 cm/s) and not in lower baPWV group (<1800 cm/s) (Ishiyama et al. 2021). To improve this negative synergistic cycle, an improvement of each component is necessary. Diabetes is one of the risk factors of SHATS. Therefore, in patients with diabetes, the exaggerated BPV is not fully absorbed by vessels but rather is transmitted to organs, causing damage. In diabetic patients, evidence that glucose variability confers a risk of macrovascular and microvascular events has accumulated (Hirakawa et al. 2014; Jang et al. 2019). Forms of glucose variability such as postprandial hyperglycemia have been shown to cause an increase of oxidative stress and result in injury to endothelial cells and artery damage (Monnier et al. 2006).

As we discussed above, day-by-day HBPV would be useful as a biomarker of DKD. On the other hand, the evidence about day-by-day HBPV as a risk factor is limited, in part because there is insufficient evidence about specific treatments for day-by-day HBPV (Fig. 6). For example, one study showed that a fix-dose combination of

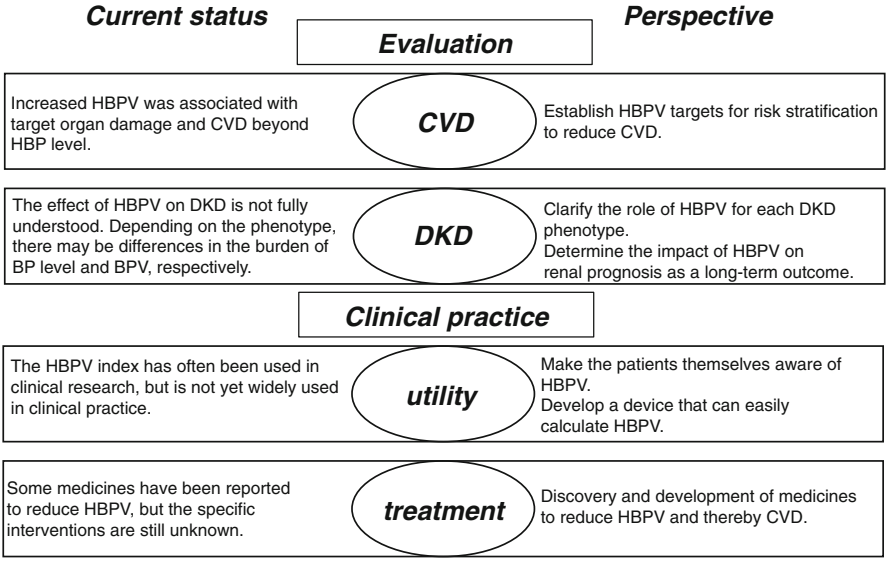

Fig. 6 Current status and perspectives on HBPV. HBPV is associated with target organ damage and CVD beyond the HBP level. However, HBPV is not yet widely used in clinical practice. It is important to accumulate evidence for practical use and to reduce CVD events in the future

cilnidipine and valsartan reduced SD, CV, and ARV of morning HBP (Kario et al. 2021b). It is anticipated that future studies will establish treatments that reduce day-by-day HBPM and thereby the incidence of target organ damage and CVD.

Perspectives on HBPM

ICT-based multisensor home and ambulatory BP monitoring (IMS-ABPM) has been developed (Kario et al. 2017) and would provide various benefits to healthcare providers and patients (Kario et al. 2017). On March 11, 2011, the Great East Japan earthquake occurred. In the disaster area, a web-based HBP monitoring system (the Disaster CArdiovascular Prevention [DCAP] Network) was introduced and used to perform BP control with web-based HBP monitoring, which achieved strict home BP control in the patients with disaster hypertension and minimized seasonal BPV over the long term (Nishizawa et al. 2017). With the current threat of COVID-19, there is a growing demand for online medicine and telemedicine. HBPM using ICT can provide both patients and clinicians with sufficient information for risk management. In addition, HBPM can be taken over several consecutive days across a period of time, while other BP measurements such as ABPM and OBP can only be taken on one specific day and may not take into account day-by-day variations. HBPM is a relatively simple-to-use and low-cost measurement. From this point of view, ICT modalities such as HBPM would be a useful tool for BP control in clinical practice for all patients (Kario 2021).

Conclusion

The results about the association between day-by-day HBPV and DKD suggest that day-by-day HBPV may be useful as a biomarker of diabetes. Therefore, the assessment of HBPV would contribute to the prevention of ESRD or CVD. HBPM using ICT management allows more appropriate risk management. On the other hand, the pathological threshold of HBPV and related specific treatments remain a challenge.

Applications to Prognosis and Other Diseases or Conditions

Applications to Prognosis

This chapter demonstrated the association between HBP or HBPV and albuminuria or renal function according to the presence or absence of diabetes (Suzuki et al. 2020). Increased mean BP levels were associated with albuminuria irrespective of the presence of diabetes, while increased day-by-day HBPV was associated with decreased renal function only in patients with diabetes. The pathogenesis of DKD is linked to hypertension, but the mechanism underlying this link is not fully understood. In addition, the pathological findings, risk of CVD, and rate of eGFR decline

differ according to the DKD phenotype. Thus, the burden of BP level and BPV may also differ according to the DKD phenotype. BPV may be useful in risk stratification and predicting prognosis for DKD.

Applications to Other Diseases or Conditions

In this chapter, we reviewed the association between day-by-day HBPV and target organ damage or CVD. Increased day-by-day HBPV was associated with new-onset CVD, independent of CVD risk factors, including mean HSBP level and target organ damage (Hoshide et al. 2018). The J-HOP study also demonstrated that a higher baPWV ($\geq$1800 cm/s) group had greater values of the day-by-day HBPV index than a lower baPWV group ($<$1800 cm/s) (Ishiyama et al. 2020). In addition, day-by-day HBPV was associated with NT-pro BNP only in the higher baPWV group, and there was an interaction between day-by-day HBPV and the NT-pro BNP according to the presence of higher baPWV (Ishiyama et al. 2020). Hemodynamic stress such as day-by-day HBPV and arterial stiffness may form a synergistic vicious cycle with each other, resulting in target organ damage and CVD (the SHATS hypothesis). Day-by-day HBPV could be a biomarker for predicting of CVD in clinical practice.

Mini-Dictionary of Terms (5–15 terms)

CVD: Includes such conditions as angina pectoris, myocardial infarction, and stroke.

Nephrosclerosis: A condition for which arteriosclerosis in the kidney is the main pathology. Hypertension is one of the most common causes of nephrosclerosis.

Non-dipper pattern: Normally, nighttime BP is 10–20% lower than daytime BP. A non-dipper pattern is often defined as a 0–10% decrease in BP from daytime to nighttime. Sometimes, the non-dipper pattern also includes the riser pattern.

Masked hypertension: A normal OBP level and high out-of-office BP level.

Target organ damage: Includes kidney and cardiovascular damages. Surrogate marker indicators for target organ damage include UACR, eGFR, the left ventricular mass index, and NT-pro BNP.

Riser pattern: A form of abnormal BPV in which nighttime BP is higher than daytime BP.

Key Facts

Key facts of albuminuria: In the past, albuminuria was measured by 24-hour urine collection, but recently, albuminuria measured by spot urine is often validated and used as an alternative measure (Bakris 2001). Because albuminuria measured by spot urine can be influenced by many factors, such as exercise and dehydration (Bakris 2001), multiple measurements are recommended. Microalbuminuria is

defined as 30 mg/gCr ≤ UACR <300 mg/gCr, and macroalbuminuria is defined as UACR ≥300 mg/gCr (Levin and Stevens 2014). Albuminuria is associated with new onset of hypertension, CVD event, and ESRD (Wang et al. 2005; Schmieder et al. 2007). In general, patients with diabetic nephropathy with macroalbuminuria experience a decline in eGFR (Nelson et al. 1996).

Key facts of OBP: OBP is the gold standard method of BP measurement in daily practice. OBP levels are associated with new onset of CVD (Umemura et al. 2019). OBP is measured twice, at least 1–2 minutes apart, and if the two readings are more than 5 mmHg apart, an additional measurement is recommended (Umemura et al. 2019). BP levels measured by OBP are variable and easily affected by the white-coat effect. Recently, a method called AOBP has been developed. This method can be performed in a quiet environment by a patient alone and can exclude the white-coat effect; it has already been used in many clinical studies (Umemura et al. 2019).

Key facts of ABPM: ABPM is available to measure BP every 15–30 minutes under free conditions on a specific day (Umemura et al. 2019). The evaluation of BP is divided into 24-hour BP, nighttime BP, and daytime BP. Diurnal BPV is assessed. In addition, by combining OBP with ABPM, masked hypertension and white-coat hypertension can be detected, and these phenotypes of hypertension have different clinical significance (Umemura et al. 2019). While ABPM is more predictive of CVD events than OBP, it also has several disadvantages, such as sleep disturbance, discomfort, and cost issues.

Key facts of eGFR: eGFR is calculated by creatinine, gender, and age (Matsuo et al. 2009). If available, inulin clearance or creatinine clearance is accurate and recommended for the evaluation of renal function. The presence of high muscle mass is associated with a higher creatinine level, which results in lower eGFR. In subjects of 45 years or older, the decline in eGFR takes place at twice the rate compared with that in younger subjects (Poggio et al. 2009). Lower eGFR is associated with increased mortality (Hallan et al. 2012).

Key facts of HBP (see main text): HBP is better than OBP in predicting CVD events (Ohkubo et al. 1998; Kario et al. 2016; Hoshide et al. 2016). HBP is usually measured in the morning and evening, with morning HBP being particularly useful for predicting CVD events (Kario et al. 2016; Hoshide et al. 2016). HBP is also highly reproducible. In general, wrist-type BP measurement has not been admitted in the guidelines, because of inadequate arterial compression and inaccuracy. HBP is usually measured in the upper arm. Recently, a validated wrist-type BP measurement device was developed.

Key facts of PWV: PWV is the speed at which arterial pulse from the heart is transmitted to the artery in waves (pulsations) (Tanaka et al. 2018). PWV is used as one of the indicators of arterial stiffness. PWV measurements are mainly evaluated by carotid-femoral PWV (cfPWV) and brachial-ankle PWV (baPWV) (Tanaka et al. 2018). Both increased cfPWV and baPWV have been associated with conventional cardiovascular risk factors (Tanaka et al. 2018). In addition, increased PWV has been shown to be a predictor of CVD events (Tanaka et al. 2018). baPWV ≥1800 cm/s (Tanaka et al. 2018) and cfPWV ≥1000 cm/s (Van Bortel et al. 2012) have often been proposed as cut-offs for high risk of CVD.

Summary Points

- In diabetes patients, ACCORD-BP showed that lowering SBP to <120 mmHg did not reduce the composite outcome (nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death) compared with lowering SBP to <140 mmHg in diabetes patients.
- Hypertension is associated with a risk of DKD, but the degree to which hypertension contributes to DKD has not been fully understood.
- The use of OBP (including AOBP) alone is not sufficient for hypertension management.
- Both HBP and HBPV have been associated with target organ damage and CVD events independent of OBP, and thus HBPM is more important for risk stratification than OBP.
- In patients with diabetes, the characteristics of blood pressure variation are nocturnal hypertension, non-dipper/riser pattern, orthostatic hypotension, and postprandial hypotension.
- DKD phenotypes include DKD with albuminuria, lower eGFR, or both.
- CVD risk and renal outcome differ according to DKD phenotype.
- In DKD without albuminuria, eGFR generally declines at a slower rate, but in some cases, eGFR declines at a faster rate, which is related to hypertension in this pathway.
- Day-by-day HBPV is also associated with target organ damage and CVD.
- There is not enough information about the associations among HBP, HBPV, albuminuria, and renal function.
- The J-HOP study demonstrated that in diabetic patient groups, increased mean BP levels were associated with albuminuria, while increased day-by-day HBPV values were associated with decreased renal function.
- Depending on the DKD phenotype classified by albuminuria and eGFR, the burden posed by BP level and BPV in patients with DKD may be different.
- Assessment of BP using ICT management would provide more appropriate risk management.

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Methods for Measuring Blood Pressure and Applications to Diabetes

35

A Focus on Children, Adolescents, and Young Adults

Andriani Vazeou and George S. Stergiou

Contents

Introduction 730
Diagnosis of Hypertension and Blood Pressure Measurement 733
Office Blood Pressure Measurement 734
Types of OBPM 734
Devices for OBPM 735
Number of Measurements per Occasion 736
Blood Pressure Measurement Technique 737
Twenty-Four-Hour Ambulatory Blood Pressure Monitoring (Table 1) 738
Home Blood Pressure Monitoring (Table 2) 740
Applications to Prognosis 742
Applications to Other Diseases or Conditions 742
Mini-Dictionary of Terms 742
Key Facts of "Methods for Measuring Blood Pressure and Applications to Diabetes: A Focus on Children, Adolescents, and Young Adults" 742
Summary Points 743
References 743

Abstract

The prevalence of hypertension in youth with diabetes is higher than in the nondiabetic individuals. Hypertension is a major cardiovascular risk factor in diabetes and contributes to the development of macro- and microvascular complications. Blood pressure (BP) measurement is essential for diagnosing hypertension. Office BP measurement is widely used but can be misleading. Twenty-

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four-hour ambulatory monitoring is recommended for detecting white-coat, masked, and non-dipping hypertension, yet it is not easily available in primary care. Home BP monitoring is more easily accessible in primary care, yet it is scarcely investigated in children, particularly those with diabetes. Proper measurement BP methodology and devices validated specifically in children should be used with all methods. In this chapter theoretical and practical information and guidance is provided for measuring office, ambulatory, and home BP in children, adolescents, and young adults with diabetes.

Keywords

Ambulatory monitoring · Blood pressure · Clinic · Diabetes · Hypertension · Measurement · Methodology · Pediatric · Blood pressure self-measurement

Abbreviations

AAP	American Academy of Pediatrics
ABPM	Ambulatory blood pressure monitoring
BP	Blood pressure
ESH	European Society of Hypertension
HBPM	Home blood pressure monitoring
KiGGS	German Health Interview and Examination Survey for Children and Adolescents
OBPM	Office blood pressure measurement
T1D	Type 1 diabetes
T2D	Type 2 diabetes

Introduction

Hypertension during childhood is an independent predictor of adult hypertension and a major cardiovascular risk factor (Sun et al. 2007). It has been reported that over the last decades, there has been a rise in the prevalence of hypertension in children and adolescents in the general population, which has been attributed to the increase of the prevalence of obesity and changes in lifestyle, such as reduced physical activity and increased inactivity time, such as the time children spend in front of a screen (television personal computer or mobile phone) (Lurbe et al. 2016; Flynn et al. 2017; Rosner et al. 2013; Din-Dzietham et al. 2007; Muntner et al. 2004; Torrance et al. 2007; Kosti and Panagiotakos 2006; Tzotzas and Krassas 2004).

Longitudinal studies have shown that elevated blood pressure during childhood and adolescence often progresses to hypertension in adult life, the so-called "tracking" phenomenon (Bao et al. 1995; Vos et al. 2003; Kollias et al. 2012). Therefore, primary hypertension in adults has its roots in childhood and adolescence (Lurbe et al. 2016; Lurbe 2003; Flynn et al. 2017; Chen and Wang 2008).

Hypertension is a strong predictor for death and cardiovascular outcomes in adults with type 1 diabetes (T1D) and type 2 diabetes (T2D) (Petrie et al. 2018)

(Rawshani et al. 2019). It has a greater impact on cardiovascular disease in diabetic patients than in nondiabetic individuals (Stamler et al. 1993). Furthermore, hypertension in youths and young adults with diabetes increases the risk for diabetes-related complications in adulthood (Dabelea et al. 2017). Despite the remarkable improvements that have been achieved in the last decades in diabetes care and the increase in life expectancy, adults with T1D have a tenfold increase in cardiovascular disease risk, and earlier onset of it compared with the general population (Shah et al. 2020; Miller et al. 2016; Rawshani et al. 2017; Rawshani et al. 2018). Furthermore, studies in adults have shown a four- to sevenfold increase in mortality rates compared with the general population, when systolic and diastolic BPs are >130 and 90 mmHg, respectively (Orchard et al. 2001).

Like adults, children with T1D are at increased risk of developing atherosclerotic cardiovascular disease before the age of 30 years (Expert Panel on Integrated Guidelines for Cardiovascular et al. 2011). This has been attributed to differences in several cardiovascular risk factors such as hyperglycemia, dyslipidemia, obesity, smoking, and hypertension (Donaghue et al. 2018). In children and adolescents with T1D and T2D, hypertension plays a major role, not only as a factor with great impact in developing macrovascular (cardiovascular) disease but also as a risk factor associated with the development of microvascular complications, such as nephropathy and retinopathy (Donaghue et al. 2018).

The prevalence of clinical hypertension in children and adolescents in the general population is ~3.5% and that of "elevated BP" (formerly termed "prehypertension") is ~2.2%–3.5%, with considerably higher rates among overweight and obese individuals (Flynn et al. 2017).

In children with T1D, the prevalence of office hypertension has been reported higher than in those without diabetes (Knerr et al. 2008; Margeirsdottir et al. 2008). In that population the prevalence of hypertension ranges from 4 to 16% as shown in large-scale studies. However, a recent analysis of 79,849 children and adolescents with T1D (Diabetes Prospective Follow-Up Registry) showed that with the application of new AAP 2017 criteria, the prevalence of hypertension increased to 44.1% (Margeirsdottir et al. 2008; Dost et al. 2020; Rodriguez et al. 2010; Schwab et al. 2006; Fornari et al. 2020; Nambam et al. 2016; Sun et al. 2007).

As in the general population, doubling of the rate of hypertension within a decade has been reported in a recent large-scale study originating from the DPV database (Dost et al. 2020). In 2008 the prevalence of hypertension was 4% and 13.9% in prepubertal children and adolescents, respectively, and in 2013 increased to 20% (both children and adolescents) and in 2018 to 29.5% (both children and adolescents) (according to the KiGGS criteria) (Dost et al. 2020; Knerr et al. 2008; Dost et al. 2014). The authors attributed this increase to several factors, including different cutoffs for hypertension set at the 97th percentile in the first paper instead of 95th percentile in the most recent one, the possible increase of obesity or overweight prevalence and the greater awareness of hypertension (Dost et al. 2020).

In children with T2D, the prevalence of hypertension in the TODAY study was 33.8% during average follow-up of 3.9 years (Copeland et al. 2011). Male sex and higher BMI significantly increased the risk of hypertension (Copeland et al. 2011).

Similar results (36%) were shown in an Australian study within 1.3 years of T2D diagnosis (Eppens et al. 2006). Higher rates of hypertension (65%) in T2D children were found in the SEARCH study with longer diabetes duration (Eppens et al. 2006; Zeitler et al. 2018). Youths and young adults with T2D have a higher age-adjusted prevalence of hypertension than youths and young adults with T1D (35.6% vs. 14.8%) (Koebnick et al. 2020; Dabelea et al. 2017). According to a recent study for each 0.01 unit of annual increase in waist-to-height ratio, adjusted relative risk for hypertension was 1.53 (95% CI 1.36–1.73) and 1.20 (95% CI 1.00–1.43) for youths with T1D and T2D, respectively (Koebnick et al. 2020). Furthermore, youths and young adults with T2D have a higher age-adjusted prevalence of macrovascular markers, such as arterial stiffness and hypertension, than youths and young adults with T1D (Dabelea et al. 2017). It has been suggested that central obesity is the key factor which could explain the higher burden of macrovascular outcomes in youths with T2D versus those with T1D (Dabelea et al. 2017).

Early recognition and treatment of hypertension in children and adolescents with T1D and T2D are of great importance since they affect future morbidity and mortality.

The classification of hypertension largely depends on the normative data, the thresholds used, and the BP measurement methodology (Dost et al. 2020). The accuracy of BP evaluation is crucial for the reliable diagnosis and management of hypertension, and recent guidelines put considerable emphasis on their appropriate implementation (Flynn et al. 2017; Lurbe et al. 2016). Inadequate BP evaluation often leads to overdiagnosis, with consequent unnecessary investigations and long-term treatment, or underdiagnosis, with consequent undertreatment and exposure to future cardiovascular disease risk (Flynn et al. 2017; Lurbe et al. 2016; Flynn and Falkner 2017). Large studies have shown that a considerable percentage of children and adolescents with diabetes and hypertension are undertreated. Among patients with diabetes diagnosed with hypertension, only 52% were receiving ACE inhibitors/ARB therapy in T1D Exchange database, and 50% were reported in another study from the Netherlands (Nambam et al. 2016; Ahmadizar et al. 2018). Furthermore, a very low percentage of patients was receiving antihypertensive treatment in the German/Austrian DPV registry reported in two studies at different time periods (2.5% and 6.5%, respectively) (Dost et al. 2020; Schwab et al. 2006). The abovementioned low percentages suggest that there is either therapeutic inertia or underdiagnosis of hypertension, or most likely both.

In a recent study from 52 centers in the SWEET international database which hosts centers of reference and collaborative centers of children and adolescents with diabetes around the world, it was shown that most pediatric diabetes centers use adequate methodology and devices for OBPM, which was in line with the current guidelines; however, there was heterogeneity among them (Gerasimidi-Vazeou et al. 2020). The authors concluded that action should be taken for further improvement and wider implementation of the current hypertension guidelines, with great emphasis on the use of BP devices validated specifically in children (Gerasimidi-Vazeou et al. 2020). Furthermore, a questionnaire has been developed which can highlight the weaknesses in the methodology and technology used in diabetes centers and

clinics for diagnosing hypertension. In addition, this could be a useful tool in hypertension research in pediatric diabetes (Gerasimidi-Vazeou et al. 2020).

This chapter provides theoretical and practical information and guidance on the methods used for measuring office, ambulatory, and home BP in children, adolescents, and young adults with diabetes.

Diagnosis of Hypertension and Blood Pressure Measurement

Three classic methods are currently used for diagnosing hypertension: office BP measurement (OBPM), ambulatory BP monitoring (ABPM), and home BP monitoring (HBPM).

OBPM is the most well-studied method and the most widely available and used in routine clinical practice. However, hypertension diagnosis, based solely on OBPM, is often misleading (Stergiou et al. 2019). This is mainly due to the white-coat and the masked hypertension phenomena, which are common in adults and in children, but also due to the poor reproducibility of office BP measurement and observer-related factors and errors (Lurbe et al. 2016; Stergiou et al. 2019; Flynn et al. 2017; Urbina et al. 2008; Williams et al. 2018; Stergiou et al. 2002; Whelton et al. 2018b).

The gold-standard method for hypertension diagnosis in children and adolescents is the 24-hour ABPM, which however is not widely accessible in primary care (Flynn et al. 2017; Flynn and Falkner 2017; Lurbe et al. 2016).

On the other hand, HBPM is widely available and recommended for diagnosing hypertension in adults, yet in children and adolescents it is not recommended as there is little evidence on its clinical utility (Flynn et al. 2017). However, in the last 15 years, research data regarding HBPM in children are accumulating, which support its usefulness in clinical practice. It should be underlined that HBPM is much more widely available than ABPM in primary care and seems to be feasible and well accepted for the evaluation of elevated BP in children (Stergiou et al. 2019).

Hypertension in children is defined as BP equal to or above the 95th percentile for age, sex, and height, whereas in adolescents (age $\geq$13 years), it is defined as SBP $\geq$130 and/or diastolic BP (DBP) $\geq$80 mmHg. "Elevated BP" (previously known as "prehypertension") is defined as BP $\geq$90th percentile for age, sex, and height to $<$95th percentile or 120/80 mmHg to $<$95th percentile (whichever is lower), or from the age of 13 years as BP 120/$<$80 to 129/$<$80 mmHg (Donaghue et al. 2018; Flynn et al. 2017).

The updated definitions of BP categories and stages and the normative data according to age, sex, and height according to OBPM, ABPM, and HBPM are thoroughly described in the latest US and European guidelines (Flynn et al. 2017; Lurbe et al. 2016).

According to the ISPAD (International Society for Pediatric and Adolescent Diabetes) guidelines in children, BP should be measured "at least" annually, while the ADA (American Diabetes Association) and the AAP (American Academy of Pediatrics) state that in children and adolescents with diabetes, BP should be

measured at each clinical visit (Flynn et al. 2017; Flynn and Falkner 2017; Donaghue et al. 2018; American Diabetes 2019).

European guidelines also recommend that children from 3 years of age, who are seen in a medical setting, should have their BP measured (Lurbe et al. 2016). All guidelines agree that hypertension should be confirmed in multiple measurements, on at least three separate occasions (Flynn et al. 2017; Lurbe et al. 2016; Donaghue et al. 2018).

It should be underlined that recent data in adults have shown that the environmental temperature must be considered in clinical hypertension research, when evaluating or comparing BP measurements taken on different occasions throughout different seasons (Gepts et al. 2020; Stergiou et al. 2020b). Clinical trials using office or out-of-office BP measurements as efficacy measures, which last long enough to be influenced by seasonal ambient temperature change, need to be adjusted for the expected seasonal variation in BP (Gepts et al. 2020, Stergiou et al. 2020b). Seasonal BP changes are evident using all daytime BP measurement methods, but not with nighttime ambulatory BP (Kollias et al. 2020).

Similarly, studies from pediatric cohorts have shown seasonal changes in BP ranging from 3.4 to 5.9 mmHg (or 0.5–1.5 mmHg per – 1 °C difference in environmental temperature) in systolic BP with a peak in fall or winter (Stergiou et al. 2020b). Potential mechanisms and mediators of seasonal BP variation include sympathetic nervous system activation with an increase of urinary and plasma norepinephrine levels in cold season. These data strongly suggest an important effect of ambient temperature on BP in children (Stergiou et al. 2020b).

Office Blood Pressure Measurement

OBPM is an unstable measurement method affected by multiple factors, which have a major impact on its level, reproducibility, and diagnostic and prognostic value (Stergiou et al. 2018b). In childhood BP may vary considerably between office visits, even within the same visit, due to several factors related to the patient, the observer, the device, and the procedure. A recent review of 328 studies in adults identified 27 sources of inaccuracy related to the above factors (Kallioinen et al. 2017).

Types of OBPM

There are several types of OBPM, depending on the device type, conditions, observer's presence, and the number of readings (Stergiou et al. 2018b). The four main OBP types are:

(i) *Auscultatory OBPM in clinical practice*: This is a poorly standardized and highly variable method which considerably overestimates BP resulting in overdiagnosis (Stergiou et al. 2018b). Moreover, the auscultatory devices might induce a systematic error with time which is not evident without calibration (Stergiou et al. 2018b).

(ii) *Automated attended OBPM in clinical practice*: When such measurements are taken in standardized conditions (few minutes resting, no talking, three or more measurements), they give similar OBPM values as "unattended automated OBPM" (Stergiou et al. 2018b). However, in clinical practice, issues such as lack of rest period, inappropriate body and arm position, and talking during or between the measurements are common and might increase OBPM (Stergiou et al. 2018b).

(iii) *Research setting OBPM*: Carefully standardized research setting OBPM has reasonable reproducibility and association with indices of preclinical organ damage which can be quite close to those with ABPM. This method provides lower BP values than casual office measurements. Obtaining such measurements in routine clinical practice is unrealistic (Stergiou et al. 2018b).

(iv) *Unattended automated OBPM*: This standardized OBPM method obtains several automated BP measurements while the patient remains alone in the examination room and provides lower BP values than casual office measurements. However, it may not be applicable in all primary care settings, as it requires a special device, more time, and office space. Furthermore, the threshold for hypertension diagnosis is lower and rather uncertain with scarce outcome data (Stergiou et al. 2018b). This is a useful method to standardize OBPM in primary care because observer errors are avoided, talking of the patient during and between measurements is avoided, and it ensures that a standard protocol (e.g., triplicate measurement) is followed (Stergiou et al. 2018b).

It is evident that all the abovementioned OBPM types have different standardization levels, different reproducibility, different clinical relevance, and different thresholds for hypertension diagnosis (Stergiou et al. 2018b; Stergiou et al. 2018d). It has been shown that, despite the use of automated devices, which eliminate the observer errors, primary care OBPM is higher and more unstable than research setting OBPM (Tang et al. 2018).

Devices for OBPM

The European and US guidelines recommend manual auscultatory BP measurement for diagnosing hypertension in the office (Flynn et al. 2017; Lurbe et al. 2016). However, electronic devices are now widely used. According to current guidelines, if hypertension is detected by the oscillometric method, it must be confirmed by the auscultatory one (Flynn et al. 2017, Lurbe et al. 2016).

For the diagnosis and management of hypertension in children, adolescents, and young adults, only upper-arm cuff devices should be used, not wrist-cuff, finger-cuff, or cuffless devices (Flynn et al. 2017, Lurbe et al. 2016).

Current guidelines recommend using only validated devices in children and adolescents (Lurbe et al. 2016; Flynn et al. 2017) (Stergiou et al. 2017a). Children are regarded a special population for BP monitor validation, requiring separate validation studies (Stergiou et al. 2017a). There are data suggesting that an electronic

BP monitor which has been successfully validated in adults might be inaccurate in children (Stergiou et al. 2017a).

Unfortunately, in a recent paper, it was shown that only 38% of pediatric diabetes centers from the SWEET consortium use electronic OBPM devices validated specifically in children, whereas 81% use devices validated in adults. Overall, only 50% of centers use either auscultatory devices or electronic ones specifically validated for children (Gerasimidi-Vazeou et al. 2020). Action should be taken to improve accuracy of BP measurement using proper equipment at different pediatric diabetes centers and clinics all over the world.

Lists of properly validated oscillometric devices for adults and specifically for children are available on the Internet (see www.stridebp.org, www.bhsoc.org). The international nonprofit organization STRIDE BP (www.stridebp.org), which operates in affiliation with the International Society of Hypertension, the European Society of Hypertension, and the World Hypertension League, provides updated lists of accurate devices for children and adults which fulfill established criteria (Stergiou et al. 2020a; Stergiou et al. 2017a).

It should be noted that for environmental reasons, mercury sphygmomanometers are being banned from clinical use. The automated electronic (oscillometric) devices have the advantage of eliminating observer bias and error, yet they may not be accurate and require separate validation in children. Unfortunately, few of them have been adequately tested in children and proved to be accurate (Stergiou et al. 2013). Aneroid devices provide a mercury-free alternative to BP measurements by auscultation, whereas oscillometric (automated) devices are increasingly becoming the norm in clinical practice due to their ease of use (Duncombe et al. 2017). However, for the evaluation of the published evidence on the accuracy of electronic BP devices, only independent and carefully designed, validation studies that meticulously applied an established validation protocol should be taken into consideration (Stergiou et al. 2017b). In a review of 38 studies investigating the accuracy of oscillometric and aneroid BP devices compared with the mercury sphygmomanometer in children, only 3 studies compared BP using aneroid and mercury devices and found comparable results (Duncombe et al. 2017). There was great heterogeneity among published studies included in the review, which is in part attributed to differences in device brand, study setting, and observer training.

Number of Measurements per Occasion

Studies in adults have used the mean of two measurements per occasion, while the NHANES analytic guidelines recommend using the mean of the second and third blood pressure readings out of three (Egan et al. 2010). Others recommend additional measurements, if the initial is above normal (Handler et al. 2012).

The current guidelines suggest that the initial BP measurement could be oscillometric or manual auscultatory (by using a mercury or aneroid sphygmomanometer (Flynn et al. 2017).

According to the 2017 guidelines for children, it is suggested to perform two additional oscillometric or auscultatory BP measurements, if the initial one is elevated (≥90th percentile) and use their average (Flynn et al. 2017). Oscillometric BP readings, particularly initial readings, tend to be higher than auscultatory readings and thus may overestimate BP and hypertension (Handler et al. 2012). Others support the assessment of at least three repeated oscillometric measurements in a single sitting to avoid inaccurate assessment (Negroni-Balasquide et al. 2016). An interval or 1–3 min between the measurements should be allowed with auscultatory and automated method (Lurbe et al. 2016; Stergiou et al. 2021b).

It should be mentioned, however, that measuring BP in toddlers is tricky, due to difficult cooperation and issues with both auscultatory and automated methods (Gerasimidi-Vazeou et al. 2020).

Blood Pressure Measurement Technique

According to the recent US and European guidelines, BP should be measured on the right arm by using standard measurement practices, unless the child has atypical aortic arch anatomy, which of course is very rare (Flynn et al. 2017). To detect possible differences, it is recommended at first visit to measure blood pressure in both arms (Lurbe et al. 2016). In cases with considerable and consistent interarm BP difference, vascular evaluation is required, and BP of the arm with the higher value should be used (Lurbe et al. 2016).

OBP should be measured in a sitting position after 3–5-min resting, with feet flat on the floor, the back leaning against the back of the chair, in a quiet room without destruction, and without speaking during the procedure (Flynn et al. 2017).

BP measurement should always be taken with the appropriate cuff size for the individual's arm circumference (Lurbe et al. 2016; Flynn et al. 2017). The inflatable bladder length should cover 80–100% of the individual's arm circumference and the width 40% of the arm circumference (4 × 8 cm, 6 × 12 cm, 9 × 18 cm, 10 × 24 cm) (Lurbe et al. 2016, Flynn et al. 2017). A large cuff applied on a small arm (i.e., adult cuff applied in children) may result in BP underestimation by up to 30 mmHg (O'Brien et al. 2003; Iyriboz et al. 1994). On the other hand, a small bladder applied to a big arm (i.e., a small cuff applied to an obese adolescent arm) might overestimate BP by up to 30 mmHg (Iyriboz et al. 1994; O'Brien et al. 2003).

Elevated body mass index in children and adolescents is associated with an increase in the midarm circumference, requiring the use of a larger cuff (standard size or large) to obtain accurate BP measurements (Flynn et al. 2017).

For precise determination of the correct cuff size, the midarm circumference should be measured at the midpoint between the acromion of the scapula and olecranon of the elbow, with the shoulder in a neutral position and the elbow flexed to 90° (Flynn et al. 2017).

The Korotkov sound I (when the first rhythmic sound appears) is used to estimate systolic BP, and sound V (when the sounds disappear) is used for

diastolic. When the sounds persist too close or up to 0 mmHg, then the Korotkov sound IV (when the sounds become muffled) is used to estimate diastolic BP (Stergiou et al. 2018a).

Twenty-Four-Hour Ambulatory Blood Pressure Monitoring (Table 1)

It is widely recognized that OBPM alone is often insufficient for the reliable diagnosis of hypertension (Flynn et al. 2017). Twenty-four-hour ABPM is currently recommended in ESH and AAP guidelines as mandatory for the diagnosis of hypertension in children, yet it is not available in most settings (Flynn et al. 2017). A recent report from the SWEET cohort showed that only 57% of centers refer to expert hypertension centers for ABPM children with elevated BP in three office visits, as current guidelines recommend. This analysis also showed that the use of ABPM is often inadequate, which could be attributed to the lack of standard strategy, or lack of specific knowledge, since children with elevated BP were referred to expert centers, which eventually decide for using ABPM (Gerasimidi-Vazeou et al. 2020).

ABPM has superior reproducibility and association with preclinical organ damage than OBPM (O'Brien et al. 2013). Particularly in diabetic children, it can detect subtle alterations, such as slight BP increases and a blunted nocturnal BP decline, early in the course of the disease, while OBPM might still be normal (Lurbe et al. 2002). It has been shown that in adolescents with T1D, an increase in nighttime systolic ABP preceded the development of microalbuminuria (Lurbe et al. 2002). A study in adolescents and young adults with T1D without hypertension or overt proteinuria showed that nighttime systolic ABP gave the strongest association with albuminuria (Stergiou et al. 2009), whereas another study in adolescents with diabetes T1D showed nighttime systolic ABP to be associated with arterial stiffness and microalbuminuria (Duzova et al. 2019).

According to the US guidelines, if OBP remains at stage 1 hypertension level after three office visits, ABPM should be performed (if available) (Flynn et al. 2017). ABPM is also useful after treatment initiation for the evaluation of resistant hypertension, for the assessment of 24-hour BP control in children with target organ damage, and when overtreatment is suspected (Flynn et al. 2017). Moreover, ABPM is mandatory for the detection of "white-coat hypertension," "masked hypertension," and the "non-dipping" pattern (Flynn et al. 2017; Urbina et al. 2008; Lurbe et al. 2016;

Table 1 Methodology for 24-hour ambulatory blood pressure monitoring

Use an automated upper-arm cuff device validated specifically in children
Use cuff of appropriate size according to individual's arm circumference
Perform on a routine day
Straighten and relax the arm when measuring BP
Calculate awake and asleep BP according to the individual's sleeping times

Sorof et al. 2001; Sorof 2000; Sorof and Portman 2000; Stabouli et al. 2005). Studies in general population with samples of children and adolescents using either ABPM or HBPM showed that when office BP measurements are used for screening, one of two children with elevated office BP appear to have white-coat hypertension, while many children with masked hypertension will be missed (Stergiou et al. 2018c). Even with carefully taken OBPM in a hypertension clinic, white-coat hypertension is as common as sustained hypertension, and children with masked hypertension are often missed (Stergiou et al. 2018c). Furthermore, ABPM has the potential to improve the detection of children at increased cardiovascular risk, but unfortunately, to date, ABPM has not been widely adopted, and robust normative values are lacking (Flynn 2011).

The US guidelines provide clear recommendations for using ABPM to confirm the need of treatment initiation in untreated individuals or treatment titration in treated ones (Flynn et al. 2017).

The European guidelines provide similar recommendations for out-of-office BP monitoring, yet they give two options for diagnosing hypertension: based on repeated OBPM using an auscultatory or electronic (oscillometric) device on several visits and on ABPM provided that these measurements are logistically and economically feasible (Lurbe et al. 2016).

An ABPB device that has been validated specifically in children should be selected, together with a cuff of appropriate size according to the individual's arm circumference (Flynn et al. 2017). Few ambulatory monitors have been successfully validated in children (lists of validated devices may be found at www.stridebp.org and www.bhsoc.org). The accuracy of automated oscillometric BP measuring devices in children is imperfect, and accuracy is not any better for ABPM than it is for home or office monitors (Stergiou et al. 2017a).

ABPM should be performed on a routine, preferably school day (Stergiou et al. 2013). The results are evaluated by calculating the 24-hour, daytime, and nighttime BP average (Stergiou et al. 2013). Actual awake and asleep time should be calculated according to the individual's diaries during 24-hour ABPM (Stergiou et al. 2013). As in the case of OBPM, ABPM in children is evaluated by using percentile normalcy tables according to gender and height or age based on a single study, which used an oscillometric device (Spacelabs 90,207) which appeared to have questionable accuracy in two validation studies in children (Kronish et al. 2017) (Stergiou et al. 2018c). Daytime and/or nighttime ambulatory blood pressure values between the 90th and 95th percentile are considered borderline, while values above the 95th percentile suggest hypertension (Stergiou et al. 2018c).

ABPM is unfortunately limited to hypertension specialists – mainly in pediatric nephrology centers (Flynn 2011; Ostchega et al. 2017). Barriers to performing ABPM in primary care have been recognized. In a recent survey in the USA, the top-ranked barriers in adults were challenges in accessing ABPM and cost. Indeed, the cost of the devices is high, ABPM is inadequately reimbursed in many countries, and there are concerns about the willingness or abilities of patients to successfully perform ABPM and its accuracy and benefits (Kronish et al. 2017; Stergiou et al. 2018c). There are still gaps in access and knowledge regarding the optimal implementation of ABPM in children (Flynn et al. 2017).

Home Blood Pressure Monitoring (Table 2)

Although in the adults HBPM is recommended as a reliable alternative to ABPM for the evaluation of both untreated and treated individuals (Stergiou et al. 2021b; Parati et al. 2021), in children the evidence on HBPM is limited, and therefore this method is not recommended for decision-making in pediatric hypertension (Stergiou et al. 2021b; Flynn et al. 2017). According to AAP recommendations, HBPM may be a useful adjunct to OBPM and ABPM after hypertension has been diagnosed (Flynn et al. 2017).

However, 70% of pediatric nephrologists prefer HBPM in the evaluation of children with hypertension or renal disease, and 64% of them consider it as more important than OBPM (Bald and Hoyer 2001; Woroniecki and Flynn 2005). HBPM appears to be a reliable screening tool for white-coat hypertension and is also a useful as a realistic alternative for clinical practice where often ABPM is not available (Stergiou et al. 2018c). Two studies comparing HBPM against ABPM (taken as reference method) for detecting white-coat hypertension in children showed high sensitivity and specificity (74–89% and 91–92%, respectively) which are in line with studies in adults (Whelton et al. 2018a).

HBPM is also useful for the long-term follow-up of children treated for hypertension (Stergiou et al. 2018c). In this case HBPM appears to be more suitable, acceptable by children and adolescents, and cost-effective than ABPM (Stergiou et al. 2013, 2018c; Parati et al. 2008).

In addition to the abovementioned indications, the European guidelines recommend HBPM in conditions where strict BP control is mandatory (high-risk patients) and in clinical trials (Lurbe et al. 2016).

For masked hypertension, the AAP guidelines repeatedly emphasize the usefulness of ABPM, yet most of such children are unlikely to be subjected to ABPM due to their low office BPs (Flynn et al. 2017). In contrast to studies in adults, two studies in children have shown that HBPM has high specificity (92–96%) but low sensitivity (36–38%) in detecting masked hypertension (Parati et al. 2008); therefore, for the diagnosis of masked hypertension in children, ABPM is recommended (Stergiou et al. 2008; Wuhl et al. 2004).

A recent study showed that in the SWEET centers, the HBPM methodology has great heterogeneity and is frequently inadequate, and only 39% of centers reported using validated electronic HBPM devices (Gerasimidi-Vazeou et al. 2020). However, there are barriers for utilizing HBPM in primary care as well, such as using

Table 2 Methodology for home blood pressure monitoring

Use an automated upper-arm cuff device validated specifically in children
Use cuff of appropriate size according to individual's arm circumference
Monitor for 6–7 days (not less than 3)
Take duplicate morning and evening measurements after 5-min sitting rest
Avoid talking during and between measurements
Evaluate the average of all readings after discarding those of the first day

devices validated in children, following the appropriate measurement procedure, reliability of reported BP readings, out-of-pocket costs of the devices, and time needed to instruct and supervise patients (Stergiou et al. 2018c; Kronish et al. 2017).

Automated oscillometric upper-arm cuff devices are recommended for HPBM. Devices that have been successfully validated specifically in children should be used with a cuff size appropriate for the child's arm circumference. The inflatable bladder length should cover 80–100% of the individual's arm circumference and the width 40–50%, or otherwise according to the manufacturers' instructions supported by validation study. Few automated devices for HBPM have been successfully validated in children (updated lists of properly validated devices may be found at www.stridebp.org and www.bhsoc.org). Wrist devices have not been validated in children and should not be used, as well as cuffless BP devices (Stergiou et al. 2018c). Measurements should be performed by parents in young children, or self-measurements in adolescents. HBPM should be monitored on 6–7 routine school days, in a quiet room, with back supported, arm resting at heart level, feet flat on the floor, and avoid talking during and between measurements (Stergiou et al. 2018c). Duplicate morning and evening measurements should be taken on each day (before drug intake if treated) after 5-min sitting rest and 1 min between readings (Stergiou et al. 2018c).

For the interpretation of HBPM results, the average of all measurements should be calculated after discarding the first day and evaluated using the normalcy table for home blood pressure in children after a study of 778 children aged 6–18 years, in a Greek school (Stergiou et al. 2007). Average home BP above the 95th percentile suggests hypertension (Stergiou et al. 2007).

A recent study suggested that the reproducibility of home BP in children and adolescents is similar to 24-hour ABPM and superior to OBPM and daytime or asleep ABPM (Stergiou et al. 2021a). The authors concluded that these findings have major implications in diagnosing hypertension in children in clinical practice by using ABPM and HBPM and in designing clinical research trials in pediatric hypertension (Stergiou et al. 2021a). It has also been suggested that HBPM and ABPM give comparable associations in young individuals with preclinical organ damage, which are superior to those by OBPM (Zeniodi et al. 2020).

Novel HBPM devices allow automated BP monitoring during nighttime sleep, and recently a single study showed that nighttime HBPM is feasible in children (Stambolliu et al. 2021). In this study the agreement between nighttime HBPM and ABPM in identifying individuals with nighttime hypertension ($\geq$95th percentile for nighttime ABP) was 82% ($\kappa = 0.49$; $P < 0.01$) and non-dippers 57% ($\kappa = 0.19$; $P = 0.03$). The authors concluded that in children and adolescents, nighttime HBPM gives similar values with nighttime ABPM and similar associations with indices of preclinical organ damage (Stambolliu et al. 2021).

It should be noted that, in contrast to adults, whose home BP and daytime ambulatory BP have similar levels, children and adolescents have considerably higher daytime ambulatory BP levels, probably due to the high level of physical activity of the young individuals during the day (Stergiou et al. 2018c).

Applications to Prognosis

Compliance of the pediatric diabetes centers and clinics with the recent US and European guidelines for the diagnosis of hypertension in children and adolescents will facilitate the accurate diagnosis of hypertension to initiate early treatment.

Furthermore, use of validated devices for measuring BP in pediatric diabetes centers and clinics will improve the accuracy of hypertension diagnosis. Improvement of BP measurement accuracy will minimize overdiagnosis of hypertension, with consequent unnecessary investigations and long-term treatment, or underdiagnosis, with consequent undertreatment and exposure to future disease risk.

Applications to Other Diseases or Conditions

The accurate BP measurement for the diagnosis of hypertension in children and adolescents and young adults with diabetes will improve future morbidity and mortality from cardiovascular disease and microvascular complications of diabetes in adulthood.

Mini-Dictionary of Terms

White-coat hypertension: elevated office and low out-of-office BP (ambulatory and/or home)

Masked hypertension: low office and elevated out-of-office BP (ambulatory and/or home)

"Non-dipping" pattern: nighttime (asleep) BP decline $<10\%$ compared to daytime (awake) levels

Key Facts of "Methods for Measuring Blood Pressure and Applications to Diabetes: A Focus on Children, Adolescents, and Young Adults"

- Hypertension affects a large proportion of children, adolescents, and young adults with diabetes.
- A considerable proportion of children and adolescents with hypertension remains untreated due to misdiagnosis or physicians' inertia.
- Proper methodology for BP measurement according to the recent US and European guidelines is imperative for the accurate diagnosis of hypertension.
- Only devices specifically validated for children should be used for the diagnosis of hypertension in pediatric diabetes centers and clinics.
- Action should be taken to improve compliance of diabetes centers and clinics with the recent guidelines to improve the accuracy of hypertension diagnosis and minimize future morbidity and mortality risk.

Summary Points

- The accuracy of BP measurement and the use of appropriate BP measurement methodology and devices in children and adolescents with diabetes are of utmost importance for the accurate diagnosis and management of hypertension.
- OBPM is the most widely used method in clinical practice; however, hypertension diagnosis, based solely on OBPM, is often misleading.
- ABPM is currently considered mandatory for the diagnosis of hypertension in children; however, it is not accessible in most settings in primary care.
- HBPM can facilitate the diagnosis of hypertension when ABPM is not available and can be particularly useful in following children treated for hypertension.
- More research on HBPM in children is urgently needed.

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Carotid Bodies: Use of Chemosensitivity as a Biomarker in Prediabetes

36

Silvia V. Conde, Marlene Lages, Maria P. Guarino, and Joana F. Sacramento

Contents

The Carotid Bodies 753
Role of CB in Metabolic Diseases 755
CB Dysfunction in Prediabetes and Type 2 Diabetes 755
What Is the Trigger for CB Dysfunction in Metabolic Diseases? 757
Evaluation of CB Chemosensitivity in Humans 758
Evaluation of Hypertonicity of Peripheral Chemoreceptors 759
Evaluation of Hyperreflexivity of Peripheral Chemoreceptors 760
Biomarkers of Prediabetes 761
Hyperinsulinemia and Obesity as Risk Factors for Prediabetes 761
Early Diagnosis of Prediabetes 761
CB Chemosensitivity as a Biomarker of Prediabetes 764
Evaluation of CB Hypertonicity by the Dejours Test 764
CBmeter: A New Medical Device for Early Detection of Prediabetes 764
Future Perspectives 767
Applications to Prognosis, Other Diseases, or Conditions 768
Mini-Dictionary of Terms 768
Key Facts of Carotid Bodies: Use of Chemosensitivity as a Biomarker in Prediabetes 769
Summary Points 769
References 770

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Abstract

The carotid bodies (CBs) play a role as metabolic sensors being deeply involved in the genesis of dysmetabolic states. CB activity was shown to be increased in prediabetes and type 2 diabetes animal models and also in prediabetic patients. Furthermore, abolishment of CB activity, via carotid sinus nerve surgical resection, both prevents and reverses pathological metabolic disease features in animal models.

Herein, we review in a concise manner the pathways linking CB chemoreceptor dysfunction to the pathogenesis of metabolic diseases and describe the methods available to evaluate CB chemosensitivity, postulated to be directly related to metabolic dysfunction. Moreover, we describe the biomarkers used to diagnose prediabetes and introduce the CB, as a novel biomarker for its early screening. A final section is devoted to debate the applications of CB chemosensitivity evaluation, not only as a biomarker for early screening of prediabetes but also to identify subgroups of patients that will benefit from therapeutics directed to modulate CB activity.

Keywords

Carotid body · Prediabetes · Insulin resistance · Glucose metabolism · Obesity · Carotid body chemosensitivity · Hypoxic ventilatory response · Dejours test · CBmeter

Abbreviations

ADA	American Diabetes Association
BMI	Body mass index
CB	Carotid body
CCA	Common carotid artery
CSN	Carotid sinus nerve
EASD	European Association for the Study of Diabetes
FINDRISC	Finnish Diabetes Risk Score
FiO2	Oxygen fraction in inspired air
HbA1c	Glycated hemoglobin
HF	High fat
HOMA	Homeostatic model assessment
HR	Heart rate
HVR	Hypoxic ventilatory response
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
iGlu	Interstitial glucose
IGT	Impaired glucose tolerance
IL	Interleukin
NTS	Nucleus of tractus solitarius
OGTT	Oral glucose tolerance test

OSA	Obstructive sleep apnea
RR	Respiratory rate
SNS	Sympathetic nervous system
SpO_2	Peripheral oxygen saturation
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

The pathophysiological mechanisms underlying the genesis and progression of metabolic diseases, such as obesity and type 2 diabetes (T2D), are complex and go beyond the undoubtedly important triumvirate of an increasingly sedentary lifestyle, changes in diet, and genetic predisposition. The nervous system, both centrally and peripherally, plays a role in the generation and maintenance of these diseases (Ruud et al. 2017), with the parasympathetic and sympathetic branches of the autonomic nervous system becoming deregulated at target key efferent organs. A particular emphasis has been given to the overactivation of the sympathetic nervous system (SNS) as a major contributor to these dysmetabolic states (Lambert et al. 2010, 2015; Thorp and Schlaich 2015), with the dysfunction of the carotid body (CB), a sensory organ located in the neck that regulates sympathetic activity, emerging as a new pathological mechanism behind the emergence of metabolic diseases (Conde et al. 2017b, 2018, 2020).

The Carotid Bodies

The CBs are paired chemoreceptor organs, located in the bifurcation of the common carotid artery into the internal and external branches, an area of high blood flow (Fig. 1). Receiving blood supply from a small branch of the external carotid artery, the CB is the organ with the largest blood flow of the entire organism, oscillating between 1.5 and 2 l/100 g/min (for a review see Gonzalez et al. 2010). In agreement, the density of capillaries within the CB represents 25–33% of the surface of histological sections obtained from organs perfused at normal (80–100 mmHg) pressure.

The CBs are synaptically connected with their sensory nerve, the carotid sinus nerve (CSN), which projects to petrosal ganglion and joins the glossopharyngeal nerve before it enters the cranium, more specifically in the nucleus of tractus solitarius (NTS) at the medulla oblongata. The CSN also integrates filaments of the vagus nerve and sympathetic innervation proving efferent parasympathetic and sympathetic influence over the CB (Gonzalez et al. 1994).

Classically, the CBs are defined as sensors of changes in arterial blood gases such as O_2 and CO_2, but they also sense alterations in blood pH. Hypoxia, hypercapnia, and acidosis activate the CB leading to the release of neurotransmitters that act on the CSN either to generate action potentials or to inhibit its activity (Gonzalez et al. 1994). The action potentials generated postsynaptically are integrated into the NTS to induce cardiorespiratory responses aimed at normalizing blood gases, via hyperventilation (Gonzalez et al. 1994), and at regulating blood pressure and cardiac performance, via activation of the sympathetic nervous system (Marshall 1994) (Fig. 1).

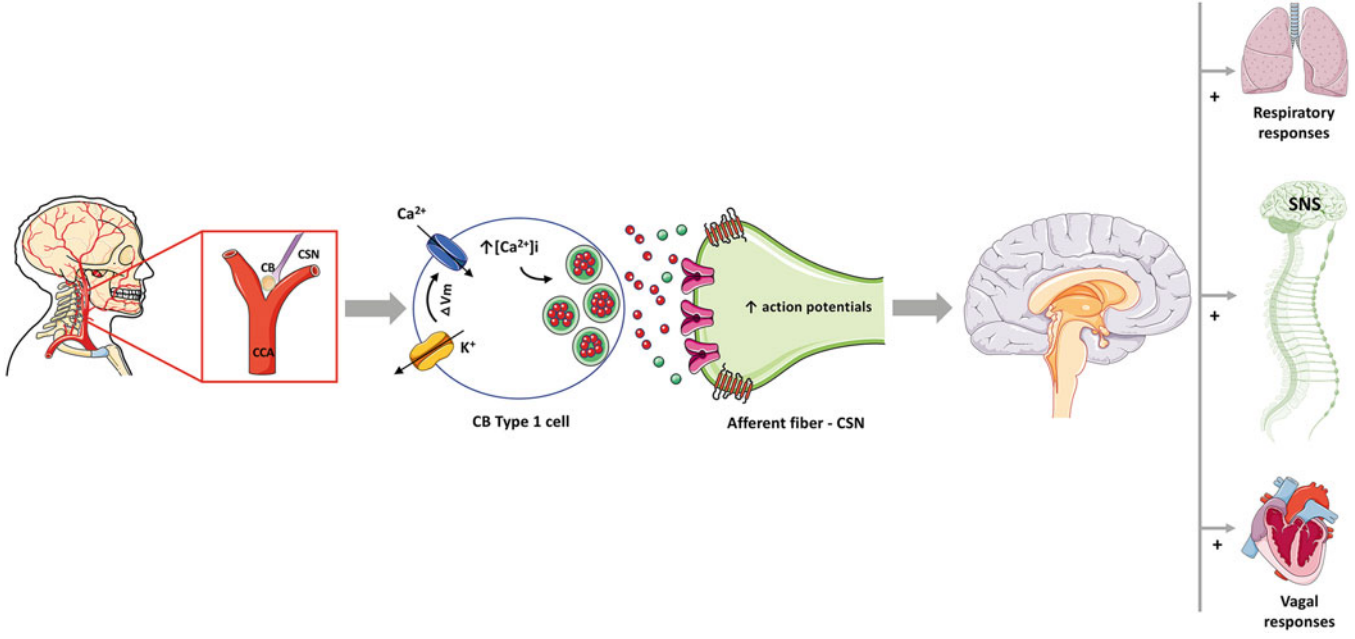

Fig. 1 ***Schematic representation of carotid body (CB) location, its mechanisms of chemotransduction, and the chemoreflexes elicited upon activation.*** CB is located bilaterally in the bifurcation of the common carotid artery (CCA). CB information is integrated within the brain through its sensitive nerve, the carotid sinus nerve (CSN). Type I cells are the chemosensory unit of the CB. Upon stimulation, for example, in response to low oxygen levels, there is the closure of K^+ channels that originates membrane depolarization and the opening of Ca^{2+} channels. The influx of Ca^{2+} into the type I cells leads to an increase in cytosolic Ca^{2+} concentrations and sequentially to the exocytosis of neurotransmitters that act postsynaptically at the CSN to increase its activity. Outputs of CB activation include cardiorespiratory chemoreflexes and the activation of sympathetic nervous system (SNS)

The CB is organized into glomeruli, clusters of cells in close contact with an abundant network of capillaries and connective tissue. Each glomerulus contains chemoreceptor cells, also known as glomus or type I cells, derived from the neural crest, and synaptically connected with the sensory nerve endings of the CSN (Gonzalez et al. 1994). These cells are enveloped by type II cells or sustentacular cells. While in the past these type II cells were thought to have a merely supportive role for type I cells, recently it was demonstrated that type II cells exhibit properties of stem cells that proliferate and differentiate into new type I cells in response to hypoxia (Pardal et al. 2007). CB type I cells contain secretory vesicles with several neurotransmitters, catecholamines (dopamine and norepinephrine), serotonin, acetylcholine, neuropeptides (substance P and enkephalins), and ATP, but also modulators, such as adenosine (for a review see Conde et al. 2017a). Type I cells express several voltage-ligand-gated ion channels and contain voltage-dependent Na^+, Ca^{2+}, and K^+ currents (López-Barneo 2018).

Role of CB in Metabolic Diseases

Metabolic syndrome, T2D, and obesity are metabolic diseases that affect millions of individuals worldwide (IDF 2021; WHO 2021). The CBs have metabolic sensing functions, being involved in the regulation of glucose homeostasis and peripheral insulin sensitivity (for a review see Conde et al. 2018; Joyner et al. 2018) and in the control of lipid and carbohydrate metabolism (for a review see Conde et al. 2014, 2017a).

CB Dysfunction in Prediabetes and Type 2 Diabetes

In the last decade, a lot of data has been generated supporting CB dysfunction as a new pathological mechanism associated with metabolic diseases and that CB/CSN modulation may be a new therapeutic target for treating these conditions (Fig. 2). Our group has shown that the abolishment of CB activity, through CSN resection or via bilateral electrical neuromodulation of the CSN in rodents, prevents the development of insulin resistance and glucose intolerance induced by hypercaloric diets and reversed dysmetabolism in animal models of metabolic diseases (Ribeiro et al. 2013; Sacramento et al. 2017, 2018). Additionally, CSN resection normalized the heightened sympathetic activity observed in metabolic disease animal models, measured through electrophysiological recordings at the superior cervical chain (Cracchiolo et al. 2019a); assessment of plasma and adrenal medulla catecholamines, and analysis of heart rate variability (Ribeiro et al. 2013; Sacramento et al. 2017). In agreement with these preclinical data, 20 sessions of hyperbaric oxygen therapy, an intervention that dramatically reduces peripheral chemoreceptor activity (Lahiri and DeLaney 1975) was shown to improve fasting glucose and postprandial glucose levels in T2D patients (Vera-Cruz et al. 2015).

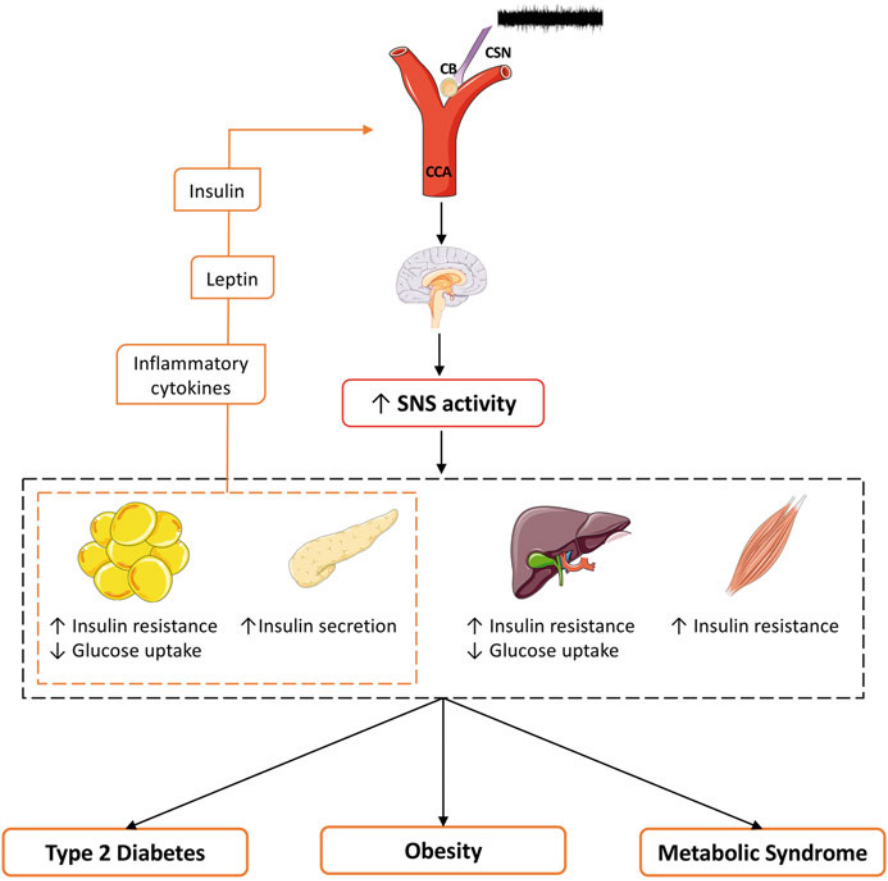

Fig. 2 ***Schematic representation of the vicious cycle involving carotid body (CB) dysfunction and the development and maintenance of metabolic diseases.*** Increased levels of insulin, leptin, and inflammatory cytokines induced by hypercaloric diets promote an increase in the CB chemosensitivity leading to an augment in sympathetic nervous system (SNS) activity that promotes metabolic dysfunction in adipose tissue, pancreas, liver, and skeletal muscle, contributing to the development of metabolic diseases, as type 2 diabetes, obesity, and metabolic syndrome

In the same line of evidence, prediabetes patients exhibited higher CB activity – measured as the cardiorespiratory response to transient hyperoxia, also known as the Dejours test (please see section "Evaluation of Hypertonicity of Peripheral Chemoreceptors") – that correlates with fasting insulin and insulin resistance (Cunha-Guimaraes et al. 2020). Also, CB dysfunction, ascertained as CB weight, % of CB chemoreceptor cells, CB tyrosine hydroxylase activity, CB release of catecholamines, CSN activity, and ventilatory parameters, was found to be higher in animal models of prediabetes and T2D (Ribeiro et al. 2013; Dos Santos et al. 2018; Cracchiolo et al. 2019a, b). This increase in CB weight and % of type I cells in metabolic disease animals is in accordance with an augment of 20–25% in the size of CB size found in patients with diabetes mellitus (Cramer et al. 2014).

Supporting the hypothesis that CB dysfunction is associated with metabolic diseases, Paleczny et al. (2016) showed that overweight or obese men exhibit CB-mediated increase in blood pressure, without changes in respiratory and heart rate responses, effects that correlated with hyperinsulinemia and insulin resistance.

Altogether, the data suggest that CB dysfunction may be related to metabolic mediator signaling pathway abnormalities particularly those that affect the autonomic nervous system such as insulin and leptin.

What Is the Trigger for CB Dysfunction in Metabolic Diseases?

Insulin

In 1962, Pereda and collaborators (Pereda et al. 1962) observed that insulin administration into the carotid artery elicited a higher increase in sympathetic activity than the systemic administration of insulin in anesthetized dogs, suggesting a possible role for systemic insulin in SNS activity. Later evidence suggested that insulin indeed might act on the CB, not only to regulate SNS tonus but also to induce changes in ventilatory parameters. Indeed, both systemic and intracarotid insulin administrations increase ventilation in a dose-dependent manner, both in animals (Bin-Jaliah et al. 2004; Ribeiro et al. 2013) and humans (Ward et al. 2007; Barbosa et al. 2018). This effect is independent of hypoglycemia as it was maintained during an euglycemic clamp (Ribeiro et al. 2013; Barbosa et al. 2018) with stable plasma glucose levels and totally due to an effect on the CB since it was lost in CSN-resected animals (Ribeiro et al. 2013). In agreement, insulin receptors were demonstrated to be present in the whole CB and to become phosphorylated in response to insulin (Ribeiro et al. 2013). In physiological concentrations, insulin also elicited a neurosecretory response in type I cells, evaluated by the augment in intracellular calcium levels and by the release of dopamine and ATP from the whole CB (Ribeiro et al. 2013). Additionally, Vidal et al. (2019) described that intracarotid administration of insulin stimulates hepatic glycogenesis, confirming that glucose homeostasis is modulated by insulin action on the CB.

The strong scientific evidence that insulin activates the CBs in animal models combined with the findings that CB activity correlates with fasting insulin and insulin resistance in prediabetes patients is a strong supporter of the hypothesis that hyperinsulinemia is one of the main stimuli contributing to CB dysfunction present in metabolic diseases.

Leptin

Leptin is an adipocyte-derived hormone that regulates food intake and energy consumption by acting on the hypothalamus (Blüher and Mantzoros 2015). This hormone also plays a role in immunity, inflammation (Berger and Polotsky 2018), and the central control of breathing (Tankersley et al. 1998; O'Donnell et al. 1999) also modulating sympathetic activity (Eikelis et al. 2003).

Leptin and leptin receptors are present at the CBs, particularly in type I cells (Ribeiro et al. 2018; Caballero-Eraso et al. 2019; for a review see Sacramento et al. 2020). Leptin administration in rodents increases minute ventilation and hypoxic

ventilatory responses (Olea et al. 2015; Ribeiro et al. 2018; Caballero-Eraso et al. 2019), effects that are abolished by CSN denervation (Caballero-Eraso et al. 2019). Moreover, CSN resection reduces spontaneous ventilation induced by acute intracarotid leptin administration, confirming the contribution of CB for both leptin effects on basal ventilation and in response to acute hypoxia (Sacramento et al. 2020). However, chronic leptin administration for 7 days in rats augmented the ventilatory responses to hypoxia without changing resting respiratory parameters. These effects were also abolished by CSN resection (Yuan et al. 2018), suggesting opposite effects of acute and chronic leptin administration on basal ventilation. Moreover, in a prediabetes animal model, obtained by feeding the animals with high-fat (HF) diet for 3 weeks, the leptin effect on basal ventilation was smaller when compared with control animals (Ribeiro et al. 2018) and not modified by CSN resection (Sacramento et al. 2020), suggesting that leptin may be involved in the CB overactivation in the initial stages of dysmetabolism, as prediabetes and overweight, but that resistance to leptin signaling and blunting leptin responses might develop with chronic hyperleptinemia. In fact, the effect of leptin on CB activity may explain why an obese model of prediabetes exhibits higher increase in spontaneous ventilation, ischemic hypoxia-induced hyperventilation, CB weight, and tyrosine hydroxylase expression, comparing with lean model of prediabetes (Ribeiro et al. 2013).

Additionally, in obesity and T2D, there may be humoral factors, apart from insulin and leptin, contributing to the cycle that leads to CB malfunctioning. In metabolic diseases adipose tissue becomes dysfunctional (Matafome and Seiça, 2017) releasing pro-inflammatory cytokines and decreasing its physiological production of anti-inflammatory cytokines (Booth et al. 2016). CBs contain tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, and their respective receptors (Lam et al. 2012; Mkrtchian et al. 2012; Kåhlin et al. 2014), and these pro-inflammatory cytokines modulate the excitability of type I cells, the release of CB neurotransmitters, and the CSN chemosensory discharge (Zapata et al. 2011; Porzionato et al. 2013) suggesting that the pro-inflammatory state that characterizes metabolic diseases may also contribute to CB dysfunction and maintenance of dysmetabolism.

Evaluation of CB Chemosensitivity in Humans

Several techniques, *ex vivo* or *in vivo*, with different degrees of invasiveness, have been employed in anesthetized and conscious animals to evaluate CB chemosensitivity, either directly or indirectly. Expression of tyrosine hydroxylase in CB type I cells, intracellular Ca^{2+} upon CB type I cell stimulation, neurotransmitter release, and the *ex vivo* evaluation of the electrophysiological activity of the CSN have been widely used post-mortem to evaluate CB activity (e.g., Ribeiro et al. 2013; Sacramento et al. 2019). In vivo CSN electrophysiological recordings (Cracchiolo et al. 2019b) and ventilatory responses to hypoxia in both anesthetized (Ribeiro et al. 2013, Pijacka et al. 2016) and conscious animals, using plethysmographic

recordings (Quintero et al. 2016), have also been widely used. *In vitro* and *ex vivo* techniques have been also used to investigate human CB morphology and function (Ortega-Sáenz et al. 2013; Kåhlin et al. 2014); however difficulties in obtaining samples and high variability due to parameters as sex, age, and pathologies among others have limited the knowledge obtained from these post-mortem studies with the additional drawback that these techniques do not provide a real-time evaluation of CB chemosensitivity. Currently, the most reliable methods to evaluate CB chemosensitivity are based on the evaluation of cardiorespiratory responses in basal conditions, and in response to challenge tests like hypoxia.

Evaluation of Hypertonicity of Peripheral Chemoreceptors

Hypertonicity of CB chemoreceptors, i.e., increased basal activity, has emerged as one of the key pathological features associated with several sympathetic mediated diseases, such as heart failure, essential hypertension, hypertension associated with obstructive sleep apnea (OSA), and metabolic diseases – obesity, metabolic syndrome, and T2D (Conde et al. 2017b). This highlights the importance of having reliable and reproducible methods to evaluate this parameter.

Tonic CB chemosensitivity can be assessed by the Dejours test (Dejours 1962), and by the use of low doses of dopamine, based on the ability of this mediator to blunt both basal CB activity (Conde et al. 2008) and the hypoxic ventilatory response (HVR) (Welsh et al. 1978; Bascom et al. 1991; Limberg et al. 2016).

In the Dejours test, ventilatory parameters are measured while subjects breathe room air (21%O2; normoxia) followed by a short period of 100%O2 (hyperoxia) (Dejours 1962, 1963). Hyperoxia blunts CB activation and when applied during a few seconds results in a decrease in ventilation, which is as high as CB chemosensitivity (Dejours 1962, 1963). CB chemosensitivity is expressed as a percentage of change in respiratory frequency during hyperoxia in relation to the normoxic period. This method is very convenient to employ, reproducible among repetitions (Cunha-Guimaraes et al. 2020), and free of secondary factors (Dejours 1962).

Administration of low doses of dopamine can also be used to assess CB chemosensitivity, since dopamine decreases basal and evoked CB activity (Gonzalez et al. 1994; Conde et al. 2008) and blunts the ventilatory response to hypoxia (Welsh et al. 1978; Bascom et al. 1991; Limberg et al. 2016). In this test, dopamine (1–4 ug. $Kg^{-1}.min^{-1}$) is administered intravenously, and the cardiorespiratory parameters in basal conditions and in response to hypoxia are assessed (for a review see Limberg et al. 2016). In healthy volunteers dopamine reduces heart rate variability (HVR), blood pressure, and total peripheral resistance in all concentrations tested; however, when subjects were clustered into low and high CB chemosensitivity, only high doses of dopamine significantly decreased HVR (Limberg et al. 2016), which may limit the definition of a standard dose to be used for evaluation of CB chemosensitivity. This might be of particular importance when evaluating CB sensitivity in pathological conditions, since it is expected that CB activity correlates with disease severity, making it difficult to titrate dopamine doses according to disease stage.

Moreover, high doses of dopamine lead to secondary effects, such as increases in blood pressure and in respiratory frequency during spontaneous ventilation (Limberg et al. 2016). Altogether these factors may limit the use of dopamine to evaluate CB chemosensitivity and in particular for identification and characterization of subgroups of pathological conditions.

Evaluation of Hyperreflexivity of Peripheral Chemoreceptors

Hypoxic ventilatory response (HVR) has been the most used method to assess an acute response from peripheral chemoreceptors (Niewinski et al. 2014; Tubek et al. 2016). Apart from HVR, hypercapnic responses have also been used (Chua and Coats 1995; Niewinski 2016) by submitting volunteers to a switch from normoxic air (room air) to hypoxia (usually 100% N2), or hypercapnia, during a few seconds, followed by rebreathing of normoxic air. Peripheral chemosensitivity is as high as the increase in minute ventilation versus the decrease in peripheral oxygen saturation (SpO2) or increase in end-tidal PCO2.

The use of adenosine to stimulate the CBs has also been described as a method to selectively evaluate CB chemosensitivity and to assess CB ablation effects (Tubek et al. 2016). Adenosine is a short half-life purine nucleoside (Moser et al. 1989; Berne et al. 1974), produced within the CB (Conde and Monteiro 2004), involved in hypoxic CB chemoresponses (Conde et al. 2012; Sacramento et al. 2019) and stimulating ventilation in animals and humans through CB activation (Watt and Routledge 1985; Monteiro and Ribeiro 1987; Watt et al. 1987, Uematsu et al. 2000; Tubek et al. 2016). In agreement with the excitatory effects of adenosine in the CB (Conde et al. 2012, 2017a; Sacramento et al. 2019), Tubek et al. (2016) described that unilateral intracarotid stimulation of the CB with adenosine produces a dose-dependent increase in minute ventilation, systolic blood pressure, and mean arterial pressure combined with a decrease in heart rate in conscious humans, effects that are abolished by CB ablation. Moreover, the authors found that individual chemosensitivity to adenosine correlates directly with HVR, suggesting that adenosine may be used to test CB chemosensitivity (Tubek et al. 2016). However, since the effect of adenosine on ventilation is dependent on the proximity of adenosine administration to the CB (Watt and Routledge 1985; Watt et al. 1987), intracarotid adenosine administration to evaluate CB chemosensitivity is a highly invasive methodology and should not be considered as an alternative for routine assessment of CB activity.

Apart from the methods based on cardiorespiratory changes in response to challenge tests, computed tomography angiography has also been used to evaluate the size of CBs in patients (Nair et al. 2013; Cramer et al. 2014; Welch et al. 2017). CB dimension has been postulated to correlate with CB function and with the progression of sympathetic CB-mediated diseases (Cramer et al. 2014). Cramer et al. (2014) found that patients with diabetes mellitus, hypertension, and congestive heart failure exhibit 20–25% larger CBs in relation to controls, a finding that is not present in other CB diseases, such as paragangliomas. However, the use of CB size may not be directly correlated with an exaggerated CB function, since patients with

OSA, a condition in which there is an increased tonic activity of the CB combined with increased sympathetic activity (Prabhakar 2016), do not exhibit increased CB size after age adjustment (Welch et al. 2017). It remains to be established if the evaluation of CB size by computed tomography angiography or other imaging techniques might be useful for the early detection of CB dysfunction and monitoring of disease progression.

Biomarkers of Prediabetes

Hyperinsulinemia and Obesity as Risk Factors for Prediabetes

Prediabetes is already recognized as a significant metabolic state. Prediabetes includes impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or both conditions, which involves a high risk of developing T2D (ADA 2021). The dysregulation of insulin secretion or clearance results in chronically elevated levels of insulin, a condition referred to as hyperinsulinemia, which is common in people with obesity or other metabolic disorders (Thomas et al. 2019). In this situation, fasting insulin increases from normal glucose tolerance to IGT to T2D (Ferrannini et al. 2005; Thomas et al. 2019). The average risk of developing T2D increases by 0.7% per year in individuals with normal glucose levels and 5–10% per year when there is IFG or IGT (Mata-Cases et al. 2015). Besides predisposing people to a high probability of developing diabetes in the future, individuals with prediabetes also have an increased risk of developing other pathologies that are usually related to this condition, including diabetic neuropathy, nephropathy, and macrovascular complications (Tabák et al. 2012).

In addition to hyperinsulinemia, obesity is also considered a major risk factor for prediabetes and, consequently, for T2D. Currently, adipose tissue is recognized as an endocrine organ that produces and releases a range of adipokines, including TNF-α and IL-6. The increased production of these adipokines has been associated with the impairment of insulin sensitivity and the development of T2D (Khaodhiar et al. 2009).

The progression from prediabetes to diabetes is not certain, and its early identification followed by the implementation of preventive measures may reduce its onset and the subsequent risk of complications. Therefore, early diagnosis is critical in the mitigation of diabetes complications and associated comorbidities.

Early Diagnosis of Prediabetes

The number of individuals with prediabetes is expected to rise substantially and is estimated to affect 417.3 million people by 2030 and 486.1 million by 2045 according to the projections of IDF (International Diabetes Federation 2021). This exponential growth in numbers is also due to the increasing rates of obesity since a raised BMI is a well-known risk factor for developing diabetes (Zimmet et al. 2001; Algoblan et al. 2014).

Current recommendations from the American Diabetes Association (ADA) for screening are almost exclusively focused on adults who are overweight (BMI $\geq$ kg 25 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2), at least up to 45 years of age, when screening is recommended to everyone. This approach can compromise the detection of this pathology in healthy and normal-weight people (ADA 2021). Also, prediabetes and T2D may be completely symptomless, which, combined with the fact that it is generally impossible to determine the exact time of the onset of this condition, leads to an extensive pre-diagnostic period. The lack of regular screening for prediabetes and early stages of diabetes leads to glucose intolerance progress going unnoticed, and many people end up with early diabetes complications and a higher cardiovascular disease risk when their condition is finally diagnosed (Hu et al. 2002). In the years before diabetes is identified, there is a rise in cardiovascular disease events, health resources, and costs (Olson et al. 2015). Therefore, the development of effective and accurate methods for diagnosing prediabetes is required to reduce the risk of this condition progressing to diabetes with complications associated.

The screening and detection of diabetes in the early stages of the natural disease course is critical for preventive management through strategies that currently can include lifestyle modifications and pharmacological therapeutics. Several predictive models have been tested in the search for the most precise approach to detect undiagnosed diabetes. These approaches include combinations of patient-reported questionnaires with objective measures, such as but not limited to age, body mass index (BMI), glucose, and glycated hemoglobin (Lindström and Tuomilehto 2003; Wilson et al. 2007).

Questionnaires and Scales

The IDF and the European Association for the Study of Diabetes (EASD) recommend the use of simple and practical questionnaires and scales to identify individuals at increased risk of developing diabetes, necessitating closer surveillance, and to limit the proportion of the population in need of diagnostic glucose tolerance tests (Alberti et al. 2007; Tankova et al. 2011). The EASD further emphasizes that screening for diabetes potential can be efficiently performed using a noninvasive risk score combined subsequently with an oral glucose tolerance test (OGTT) in those at high risk (Cosentino et al. 2020).

Several scales have been developed and validated to stratify the risk of developing the disease (Tankova et al. 2011; Buijsse et al. 2011), such as the Finnish Diabetes Risk Score (FINDRISC) which is one of the most used scales. This tool was developed in 2001 by the Finnish National Diabetes Program, and it is based on eight questions regarding variables related to the risk of developing diabetes, and it estimates the probability of developing diabetes over the next 10 years (Schwarz et al. 2009; Buijsse et al. 2011; Mata-Cases et al. 2015). This could be an effective questionnaire to be filled by the patients as it has been successfully implemented as a simple screening tool to assess diabetes risk and diagnose previously unrecognized diabetes in several European populations (Paulweber et al. 2010). The FINDRISC questionnaire may also be important to assess modifiable risk factors that are

assessed by this scale, such as overweight and obesity. Also, abdominal fat is assessed by measuring waist circumference, which may be a better indicator of the risk of developing diabetes than just BMI (Alberti et al. 2007). The ADA developed the Diabetes Risk Test, which is another option to assess the suitability of testing for diabetes or prediabetes in asymptomatic adults based on their age, gender, weight, level of physical activity, and medical history. A score of 5 or higher indicates an increased risk for having T2D, but this questionnaire does not stratify the risk according to the different answers (ADA 2021). However, one of the main limitations of screening for prediabetes using questionnaires is the associated bias, as it is dependent on the patient's response. Also, the measures and risk index obtained with this type of method are more subjective. In contrast, the use of biochemical or physiological assessments can improve the performance and accuracy of screening models that are based on noninvasive measures (Buijsse et al. 2011; Waugh et al. 2013).

Biochemical Markers

The World Health Organization (WHO) and the ADA have guidelines for screening prediabetes based on the assessment of the levels of IGT and IFG. Both entities have defined in their guidelines the same thresholds for IGT; however, the ADA guidelines recommend a lower threshold for IFG than the WHO guidelines (WHO 2006; ADA 2021). This decision was made to attempt to improve the concordance between the IFG and IGT estimated prevalence from the two guidelines. Impaired fasting glucose is considered a marker of hepatic insulin resistance, and it is a more important predictor of diabetes risk than skeletal muscle insulin resistance described by IGT (Yip et al. 2017).

The assessment of IFG is based on the level of fasting plasma glucose, whereas IGT is determined using the 2-h plasma glucose during a 75 g oral glucose tolerance test (OGTT). Although the OGTT is considered the gold-standard test to assess glucose tolerance, generally, it is not recommended to use this test as a mass screening of prediabetes and T2D. The OGTT is an invasive procedure that requires multiple blood draws, it is expensive, and it can also be inconvenient to both the patients and healthcare providers.

Glycated hemoglobin (HbA1c) can also be used as a biomarker of prediabetes. According to the ADA, values between 5.7% and 6.4% are considered prediabetes (ADA 2021). This test has the advantage that it can be performed without the need for a fasting period, unlike the fasting plasma glucose or the OGTT. Furthermore, as this biomarker is an indirect measure of mean blood glucose values over 3 months, it eliminates day-to-day variability as a confounding factor in the assessment of IGT or IFG (Sequeira and Poppitt 2017). Nevertheless, this biomarker also presents some limitations, including moderate sensitivity and specificity, and it can also be inaccurate since HbA1c may be affected by ethnicity, hemoglobin variants, and other clinical conditions (Barry et al. 2017; Dorcely et al. 2017; Hostalek 2019). The use of a single biomarker has inherent limitations; thus, the identification of additional biomarkers should be explored, allowing a combination more likely to precisely identify those at a higher risk for developing prediabetes.

Prediabetes screening actions are an essential practice to prevent the progression of this state and to further control the prevalence of T2D and its complications. However, traditional methods involving the determination of capillary blood glucose, the isolated assessment of body weight, and other parameters did not show to be sufficiently discriminatory regarding the risk of the disease. As such, it is essential to investigate new screening methods that take into account the etiology of this pathology and that, in addition, allow detecting diabetes even in the asymptomatic stage.

The growing evidence shows that equally the early diagnosis and the adoption of therapeutic measures to improve glycemic control after the disease has set in allow for important reductions in the incidence, complications, and comorbidities of this pathology (Tuso 2014). These measures should include strategies that encompass the prevention of diabetes through the reduction of known risk factors, and the identification of groups at increased risk for developing diabetes.

CB Chemosensitivity as a Biomarker of Prediabetes

Evaluation of CB Hypertonicity by the Dejours Test

Using the two-breath Dejours test, in which patients breathe 100% O2 during two breaths after a room air breathing period, Cunha-Guimaraes et al. (2020) tested CB hypertonicity in prediabetic patients. CB hypertonicity was measured as the % of decrease in respiratory rate (RR) produced by 100%O2, being the RR decrease as higher as CB chemosensitivity. The authors found that prediabetic patients ($n = 33$), characterized by HbA1c levels between 5.7% and 6.4%, by increased insulin resistance measured through the homeostatic model assessment (HOMA), and by increased glucose intolerance through increased glycemia levels 2 h post-OGTT, showed higher decreases in RR meaning higher CB chemosensitivity than non-prediabetic volunteers (Cunha-Guimaraes et al. 2020). Moreover, increased CB chemosensitivity correlated with fasting plasma insulin levels and with HOMA index in prediabetes patients. Altogether the results suggest that CB chemosensitivity may represent a novel noninvasive functional biomarker to forecast early metabolic disease.

CBmeter: A New Medical Device for Early Detection of Prediabetes

Although knowing that evaluation of CB activity may represent a novel noninvasive functional biomarker to forecast early metabolic disease (Cunha-Guimaraes et al. 2020), the field is still lacking a noninvasive and easy-to-use equipment designed to detect changes in CB activity. Our team developed a prototype, the CBmeter, which consists of a set of modules for the acquisition of the physiological signals: a peripheral oxygen saturation sensor; a sensor for respiratory rate assessment; an ECG sensor for heart rate evaluation; a sensor for noninvasive glucose monitoring; and a module of integration of physiological signals (Fig. 3). The prototype also includes a new analytical methodology that combines the analysis of all the

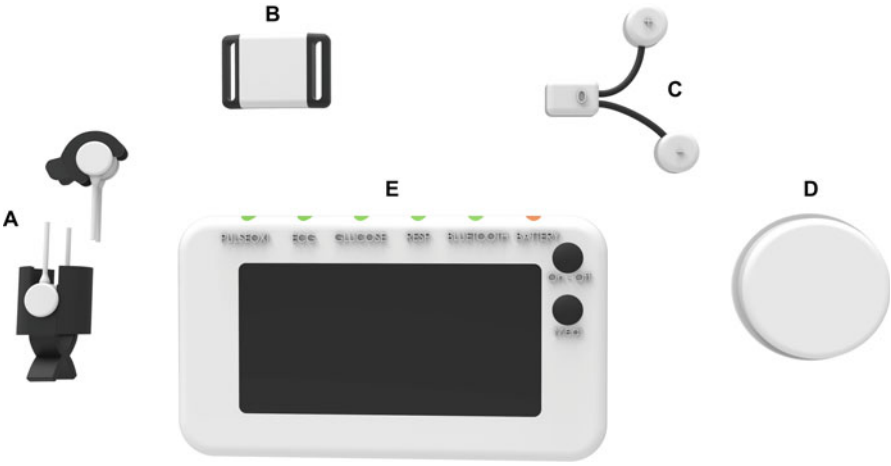

Fig. 3 ***Layout of the CBmeter prototype, a device designed to measure physiological signals related to carotid body activity.*** The prototype of the device includes a sensor to measure peripheral oxygen saturation sensor (**a**); a sensor to measure cardiothoracic movements to determine the respiratory rate (**b**); an ECG sensor to assess heart rate (**c**); a sensor for continuous glucose monitoring (**d**); and a module for acquisition and integration of physiological signals (**e**)

CB-activity indirect measurements, allowing to quantify the risk of developing metabolic disease in healthy individuals, and also monitoring the evolution of the metabolic disease in people diagnosed with prediabetes and T2D.

The CBmeter evaluates the decrease in ventilation caused by two successive inspirations of oxygen fraction in inspired air (FiO2) of 100%, a decrease that is proportional to the chemosensor activity of CBs (Wehrwein et al. 2010, 2015). The device will allow the correlation between subtle alterations of the chemoreceptors with the silent development of metabolic diseases. The evaluation of the activity of the CBs through this methodology implies the performance of two provocation tests. For one of the tests, the team developed a standard mixed meal to raise glucose levels with specific characteristics – CBmeal.

Data analysis will be performed using software designed for this purpose – CBview (Brito et al. 2018).

At the functional level, the device is simple to use, having automatic or manual adjustment mechanisms, to ensure a surface of contact or permanent access with the wearer's skin, but without causing any discomfort and restrictions on freedom of movement. Its primary functions are the continuous monitoring of physiological parameters in a noninvasive manner, heart rate (HR), RR, and SpO2, with additional modules that allow recording of other physiological parameters of interest such as glucose levels in sweat. As a noninvasive medical device, the sensors are used to take measurements on the surface of the skin. With a prototype developed by our team, we performed a clinical study on healthy volunteers ($n = 25$) recruited at the Health Services of Polytechnic of Castelo Branco and prediabetic volunteers ($n = 8$), recruited at the Hospital Centre of Leiria. CB activity was assessed by continuous

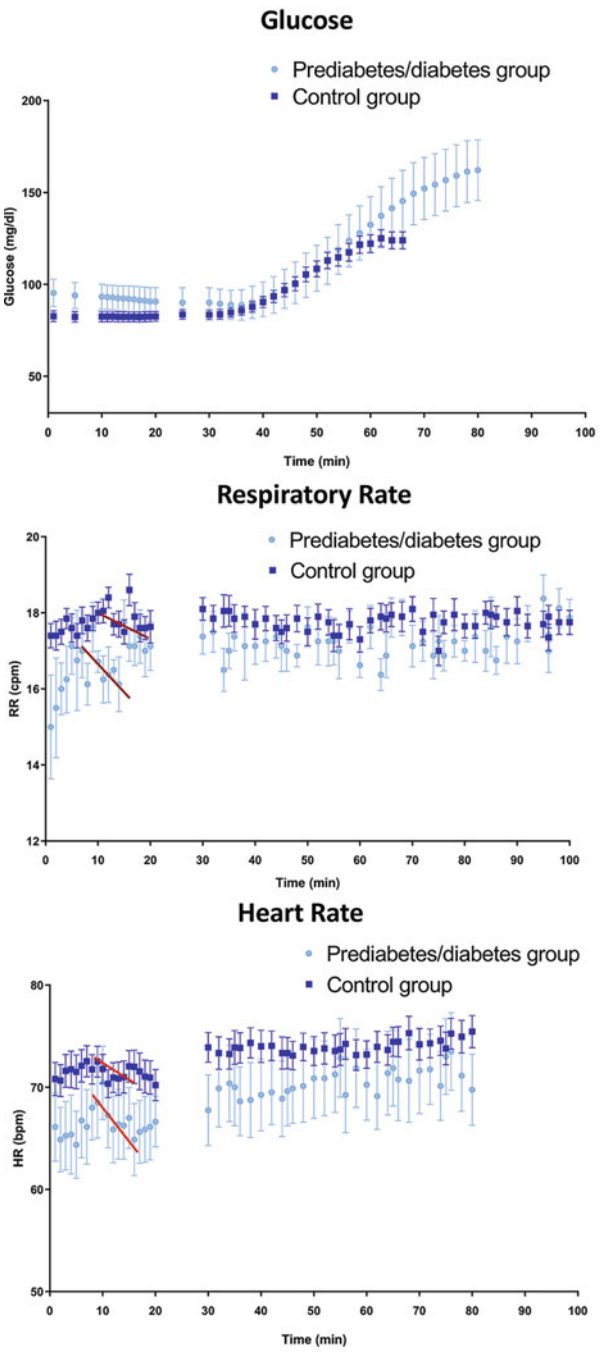

Fig. 4 ***Graphics comparing the mean values of the physiological variables evaluated with the CBmeter device between the control group and the prediabetic/diabetic group.*** Physiological

monitoring of HR, RR, SpO2, and interstitial glucose (iGlu) in response to a hyperoxic challenge through the administration for 10 s of 100% medicinal oxygen and during the intake of a standard mixed meal (65% of carbohydrates, 23% of protein, and 12% of lipids). The physiological signals were acquired with the CBmeter prototype and processed using the CBview. The results obtained with the CBmeter showed that the mean values of iGlu and HR, but not RR or SpO2, were significantly different between healthy volunteers and prediabetes patients (repeated measures ANOVA with a Greenhouse-Geisser correction, $F = 13.450$, $p = 0.006$, and $F = 6.415$, $p < 0.05$, respectively) (Fig. 4). Post hoc tests using the Bonferroni correction revealed that glucose values significantly increase after ingestion of the mixed meal in both groups ($p = 0.038$ and $p = 0.013$, respectively). Comparing clusters of RR values between healthy and prediabetes volunteers, we observe significant differences in RR values at time points corresponding to the hyperoxia challenge ($p = 0.007$) (Fig. 4b). Post hoc tests using the Bonferroni correction revealed that HR values increase after meal ingestion although the values were not statistically significant among the two groups tested (Fig. 4c). As such, the diabetes group appears to be more sensitive to oxygen in terms of both RR and HR decreases. This means that their CBs respond much more effectively to oxygen than controls, which indicates that there is overactivation of these organs. We concluded that the CBmeter detects different patterns of cardiorespiratory and metabolic variations in response to hyperoxia and to a mixed meal in healthy and prediabetic volunteers. The data confirm that CB activity is altered in prediabetes patients and that the CBmeter may be a useful tool for the early diagnosis of dysmetabolism in asymptomatic patients.

Future Perspectives

Diabetes is a worldwide problem, with a total of approximately 422 million people diagnosed, numbers projected to increase by 25% in 2030 and 51% in 2045 (Saeedi et al. 2019). Between 2000 and 2016, there was a 5% increase in the world's diabetes mortality, and in 2019, about 1.5 million deaths were directly caused by diabetes.

Early diagnosis of this pathology allows timely intervention, reducing the risk of developing complications and reducing the mortality rate. In addition to health gains, early diagnosis also reduces the high costs and the burden on healthcare professionals. As such, it is considered that the development of new early

Fig. 4 (continued) variables were assessed: at baseline (0–10 min); after an hyperoxic 100% oxygen challenge at t = 10 min; and after a mixed meal challenge performed between 20 and 30th minute. Left panel shows mean values of glucose excursion curves in control and prediabetic patients ($F = 13.450$, $p = 0.006$; repeated measures ANOVA with a Greenhouse-Geisser correction). Middle panel shows mean values of respiratory rate (RR) ($F = 2.765$, $p = 0.080$; repeated measures ANOVA with a Greenhouse-Geisser correction). Right panel shows mean values of heart rate that were statistically significantly different when using an ANOVA with repeated measures with a Greenhouse-Geisser correction ($F = 6.415$, $p < 0.05$)

diagnostic methodology will have a high impact on society. Therefore, a methodology that provides early screening of CB dysfunction may represent a biomarker of subclinical disease development allowing timely lifestyle interventions and therapeutic approaches that prevent progression of the disease and associated comorbidities. Evaluation of CB chemosensitivity, particularly through a minimally invasive, easy-to-use, and designed for primary healthcare setting device, like the CBmeter will contribute to diminish the high numbers of diabetes incidence. Moreover, assessment of CB chemosensitivity will allow screening patients with CB-associated metabolic dysfunctions that will benefit from CB-targeted therapeutics.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter the chemosensitivity of the carotid body (CB) has been reviewed as an early biomarker for the diagnosis of metabolic diseases and to follow its progression. Chemosensitivity of the CB was shown to be increased in prediabetes patients and to be directly correlated with fasting insulin levels and with insulin resistance (Cunha-Guimaraes et al. 2020*). Preliminary studies using the CBmeter, a device that combines sensors to assess heart rate, respiratory rate, peripheral oxygen saturation, and glucose levels to determine the respiratory and metabolic chemoreflex of the carotid bodies after performing provocation tests, allowed to noninvasively associate the function of the carotid bodies with the diagnosis of endocrine disruption.*

This kind of methodology might be used to forecast not only metabolic diseases but also other diseases associated with CB dysfunction and that originates increased CB chemosensitivity, namely, essential hypertension, obstructive sleep apnea, and heart failure.

Mini-Dictionary of Terms

- **Carotid body chemosensitivity** – includes carotid body hypertonicity and hyperreflexia or one of these.
- **Carotid body hypertonicity** – increased carotid body basal activity.
- **Carotid body hyperreflexia** – increased response of the carotid body to acute stimuli, e.g., hypoxia.
- **Carotid sinus nerve** – a branch of the glossopharyngeal nerve that contains the afferent fibers of chemoreceptors in the carotid body, being these fibers integrated in the nucleus of tractus solitarius.
- **CBmeal** – a standard mixed meal composed of 65% of carbohydrates, 23% of protein, and 12% of lipids that is administered to induce an increase in blood glucose levels to assess the metabolic chemoreflex of the carotid bodies.
- **CBmeter** – a device that combines sensors to assess heart rate, respiratory rate, peripheral oxygen saturation, and glucose levels to determine the respiratory and metabolic chemoreflex of the carotid bodies after performing provocation tests.

- **CBview** – a novel software, developed using MatLab, which analyzes the records of physiological responses mediated by the carotid bodies to provocation tests.
- **Hypoxic ventilatory response** – defined as the increase in ventilation induced by hypoxia allowing the body to respond adequately to a low oxygen pressure.
- **Impaired fasting glucose** – is defined as fasting plasma glucose levels of 100–125 mg/dL (5.6–6.9 mmol/L).
- **Impaired glucose tolerance** – blood glucose levels are increased beyond normal levels, specifically glucose levels of 140–199 mg/dL (7.8–11.0 mmol/L) 2 h after a 75 g oral glucose tolerance test.
- **Oral glucose tolerance test** – is used to diagnose disturbances in glucose metabolism or insulin resistance, and it consists of the oral administration of liquid containing 75 g of glucose after overnight fasting.
- **Prediabetes** – a condition in which subjects have blood glucose levels higher than normal, but not high enough to be classified as diabetes.

Key Facts of Carotid Bodies: Use of Chemosensitivity as a Biomarker in Prediabetes

- The CB is a peripheral chemosensory organ that exhibits metabolic sensing properties, being deeply involved in glucose homeostasis.
- CB dysfunction is involved in the genesis of insulin resistance and glucose intolerance since the abolishment of its activity via the resection or electrical modulation of its sensitive nerve, the CSN, prevents and reverses the development of these pathological features in animal models of metabolic disease.
- CB activity was shown to be increased in animal models of metabolic disease.
- Prediabetes patients exhibit increased CB chemosensitivity, i.e., increased CB activity that correlates with fasting insulin levels and with insulin resistance.
- Prediabetes has a prevalence of around 28%, and it is recognized as an important metabolic state that predisposes people to a higher probability of developing diabetes in the future.
- Early screening of prediabetes allows the implementation of preventive measures that reduce the number of cases that progress to diabetes as well as the percentage of comorbidities and costs associated with this pathology.
- The CBmeter is a novel and innovative device that allows associating the function of the carotid bodies with the diagnosis of endocrine disruption, which is currently not part of common clinical practice.

Summary Points

- CB chemosensitivity is increased in prediabetes and T2D animal models and prediabetic patients, an effect abolished after CSN resection or electrical modulation in rodents.

- Hyperinsulinemia, hyperleptinemia, and inflammation contribute to CB overactivation leading to an increase in sympathetic nervous system activity promoting the development of metabolic diseases such as metabolic syndrome, obesity, and T2D.
- The evaluation of CB activity might be used as an early diagnostic tool for metabolic dysfunction and its severity and to characterize metabolic disease patients, in order to identify the subjects that will benefit from CB/CSN modulation.
- The CBmeter is a new noninvasive device that assesses interstitial glucose, respiratory rate, heart rate, and oxygen saturation in real time and synchronously enabling associating the function of the carotid bodies with the diagnosis of endocrine disruption.

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Optical Coherence Tomography Angiography for Biomarker Indices in Diabetes

37

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Contents

Introduction 778
Principles of Optical Coherence Tomography Angiography 778
Visualization Methods of Optical Coherence Tomography Angiography 779
Diabetic Retinopathy 779
Optical Coherence Tomography Angiography Features of Diabetic Retinopathy 780
Diabetic Eyes Without Clinical Retinopathy 780
Correlation with Other Biomarkers of Diabetic Retinopathy 781
Nonproliferative Diabetic Retinopathy 781
Proliferative Diabetic Retinopathy 783
Diabetic Macular Edema 783
Applications to Prognosis and Other Diseases or Conditions 784
Application to Diabetic Retinopathy Screening and Its Monitoring 784
Application to Retinal Vascular Diseases 785
Limitations of OCTA 785
Mini-Dictionary of Terms 785
Key Facts of Optical Coherence Tomography Angiography (OCTA) in Diabetic Retinopathy (DR) 786
Summary Points 786
References 787

Abstract

Optical coherence tomography angiography (OCTA) visualizes the structure and flow of retinal vasculature using motion contrast. Diabetic retinopathy (DR), commonly present in one-third of the patients with diabetes, is a major microvascular complication of diabetes mellitus caused by retinal ischemia and vascular hyperpermeability. Microvasculature changes, such as foveal avascular zone enlargement

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and decrease in vascular density of the deep capillary plexus, have been identified in diabetic eyes without clinical retinopathy. OCTA of nonproliferative DR shows morphologically well-defined microaneurysms and intraretinal vascular shunts around perfusion dropouts at the deep capillary plexus level. In proliferative DR, OCTA visualizes detailed features and extent of neovascularization in response to anti-vascular endothelial factor and laser treatment. Diabetes-induced microvascular changes can be quantified by OCTA, and significant correlations have been shown with serum biomarkers representing pancreatic β-cell function and insulin sensitivity. Compared to conventional fluorescein angiography, OCTA can be performed more safely in patients with impaired renal function and can provide high-contrast vascular images. OCTA limitations on projection artifacts, segmentation errors, and data differences between equipment remain to be addressed.

Keywords

Diabetic retinopathy · Foveal avascular zone · Neovascularization · Optical coherence tomography angiography · Vascular density

Abbreviations

DM	Diabetes mellitus
DME	Diabetic macular edema
DR	Diabetic retinopathy
FAZ	Foveal avascular zone
FFA	Fluorescein angiography
HbA1c	Glycated hemoglobin
HOMA	Homeostasis model assessment
ILM	Inner limiting membrane
NPDR	Nonproliferative diabetic retinopathy
OCT	Optical coherence tomography
OCTA	Optical coherence tomography angiography
ONH	Optic nerve head
PDR	Proliferative diabetic retinopathy
PRP	Panretinal photocoagulation
VEGF	Vascular endothelial growth factor

Introduction

Principles of Optical Coherence Tomography Angiography

Optical coherence tomography (OCT) generates the structure of the retina or anterior segments of the eye by measuring the interference of the reflected scanning beam from ocular tissues (Spaide et al. 2018). OCT angiography (OCTA) is a functional extension of structural OCT and produces high-resolution images that help visualize the structure and flow of vasculature in a noninvasive and rapid manner (Spaide et al.

2015). The principle of OCTA is to visualize the vasculature by detecting motion contrast from circulating blood cells. OCTA involves acquiring multiple B-scans from the same retinal location. Decorrelation data on motion contrast are combined into cross-sectional images, which are used to produce volumetric data.

The acquisition time for each B-scan is determined by the A-scan rate multiplied by the number of A-scans per B-scan (Spaide et al. 2018). The fly-back time is measured when the OCT beam is scanned back to its initial position. Therefore, B-scans can be repeated after a time delay, that is, interscan time, which is the sum of the acquisition time and fly-back time. The interscan time is critical for motion contrast detection. Shortening the interscan time decreases sensitivity to blood cell flow and reduces the effects of unwanted eye movements. Conversely, the sensitivity improves by increasing the interscan time.

Visualization Methods of Optical Coherence Tomography Angiography

Segmentation of volumetric data projecting OCTA over axial depth ranges enables a separate enface view of vasculature corresponding to each retinal layer and the choriocapillaris (Choi et al. 2017). However, enface OCTA images are extremely susceptible to segmentation error, especially in retinas with pathology. The cross-sectional OCTA view allows quick capturing of the depth of vascular pathology and provides precise information on flow. In addition to enface display, examining cross-sectional images helps evaluate the potential for segmentation errors and projection artifacts inherent in enface imaging.

Diabetic Retinopathy

Diabetic retinopathy (DR) is a major microvascular complication of diabetes mellitus (DM) and a predictor of other life-threatening end-organ complications (Aminian et al. 2020). DR occurs in approximately one-third of the patients with diabetes (Mohamed et al. 2007), and approximately 10% of them develop vision-threatening retinopathy (Klein 2008). Hyperglycemia causes a series of events that lead to retinal vascular endothelial dysfunction. The resulting retinal ischemia and an increase in vascular permeability enhanced by hypertension are two important common pathways underlying the development and progression of DR (Curtis et al. 2009). DR has a significant impact on the retinal vasculature; therefore, OCTA has a broad application in patients with diabetes. DR can be graded into two major stages, including early-stage non-proliferative DR (NPDR) and advanced-stage proliferative DR (PDR). Clinical grading of DR has been dependent on vascular features in color fundus photographs as well as fundus fluorescein angiography (FFA) (Flaxel et al. 2020). Characteristic signs of NPDR are retinal hemorrhage, microaneurysm, cotton wool spots, hard exudate, intraretinal microvascular abnormalities, and venous beading (Early Treatment Diabetic Retinopathy Study Research 2020). Neovascularization of the retina or iris is a

hallmark feature of proliferative DR (Stitt et al. 2016). Changes in the retinal microvasculature that occur before the first clinical signs of DR appear may play an important role as a biomarker of DM.

Optical Coherence Tomography Angiography Features of Diabetic Retinopathy

Diabetic Eyes Without Clinical Retinopathy

OCTA can reveal microvascular changes in diabetic eyes without clinical retinopathy (Fig. 1). The foveal avascular zone (FAZ) is an avascular region located in the foveal center and reflects the degree of macular ischemia in DR. Enlargement of the FAZ is confirmed using OCTA in diabetic eyes without clinical retinopathy (de Carlo et al. 2015). In addition to the FAZ expansion, a decrease in retinal vascular density can be detected, especially in the deep capillary plexus (Al-Sheikh et al. 2016; Carnevali et al. 2017; Scarinci et al. 2018). In patients with a long history of DM, a reduction in the capillary plexus has also been observed in the superficial layer before the onset of clinical signs of DR (Choi et al. 2020; Vujosevic et al. 2019). OCTA may be used as a DR screening tool before it is clinically detectable.

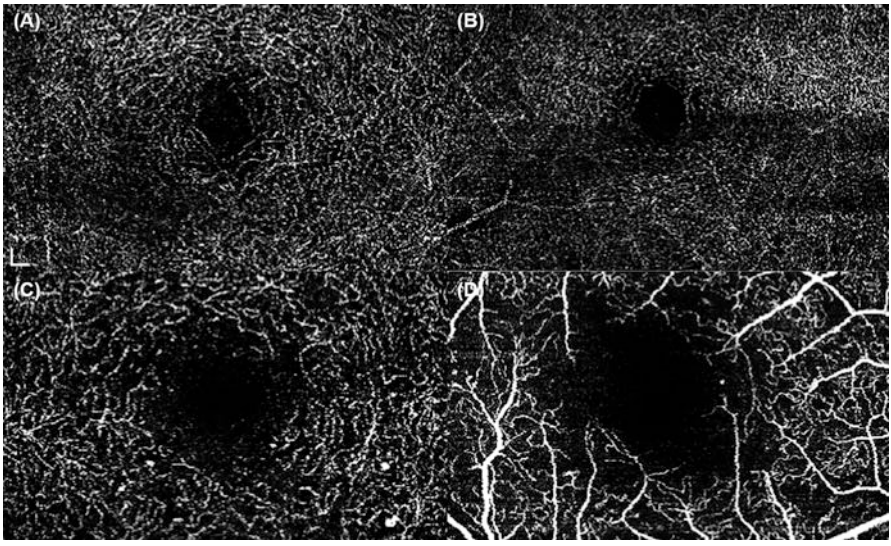

Fig. 1 Enface optical coherence tomography angiography images of the deep capillary plexus in patients with different stages of diabetic retinopathy. Segmentation slab of the deep capillary plexus is defined from the outer border of the inner plexiform layers to the outer plexiform layers. A healthy control (**a**). An eye with no clinical diabetic retinopathy (**b**) shows minimal change in the foveal avascular zone (FAZ), nonproliferative diabetic retinopathy (**c**), and proliferative diabetic retinopathy (**d**). As diabetic retinopathy progresses, FAZ expansion and microcapillary dropouts become more prominent

Microaneurysms are observed as dilated dots on the retinal capillary walls of various sizes and morphologies (Al-Sheikh et al. 2016; Dimitrova et al. 2017). Unlike FFA, OCTA can detect microaneurysms by each retinal layer, more commonly located in the deep capillary plexus rather than the superficial layer (Couturier et al. 2015). Because of the flow requirements of OCTA to detect a microaneurysm, not all microaneurysms can be detected using OCTA, and OCTA detection rates are usually lower than FFA (Matsunaga et al. 2015; Couturier et al. 2015).

Correlation with Other Biomarkers of Diabetic Retinopathy

Long duration of diabetes is one of the main factors that increases the risk of developing DR (Fong et al. 2003). However, a correlation between the duration of DM and microvascular impairment detected by OCTA has not been reported (Carnevali et al. 2017; Choi et al. 2020). The level of glycemic control and tolerance to diabetes are independent contributing factors for the development of DR. However, glucose levels, glycated albumin, and glycated hemoglobin (HbA1c), well-known serum biomarkers for glycemic control (Vujosevic et al. 2019; Jenkins et al. 2015), have limited correlation with initial microvascular changes (Choi et al. 2020). Pancreatic β-cell function and insulin sensitivity are assessed by various markers, such as C-peptide and insulin levels, and homeostasis model assessment (HOMA) values (Ludvigsson 2013; Hirata et al. 2015). Among these serum biomarkers, low insulin levels and HOMA values have been shown to have an inverse correlation with the levels of macular microvascular impairment (Choi et al. 2020).

Nonproliferative Diabetic Retinopathy

In eyes with NPDR, diabetes-induced vascular lesions can be observed in detail using OCTA (Fig. 2). Enlargement of the FAZ is detected more prominently in the deep capillary plexus than in the superficial capillary plexus (Takase et al. 2015; Di et al. 2016). OCTA can reveal the abrupt capillary margins of FAZ with more blind ends than that observed on FFA (Tam et al. 2012; Takase et al. 2015). The FAZ area is inversely correlated with central macular thickness (Lupidi et al. 2017; Samara et al. 2015). In addition to FAZ enlargement, OCTA can be used to assess the changes in non-perfusion areas caused by capillary dropout during DR progression (Couturier et al. 2015; Zhang et al. 2016). Non-perfusion areas are detected more in the superficial capillary plexus, and the severity of non-perfusion areas is related to vascular abnormalities in the deep capillary plexus, such as FAZ enlargement and microaneurysms (Couturier et al. 2015). More severe non-perfusion areas of the deep capillary plexus are associated with impairment of the outer retinal structures (Nesper et al. 2017). Adjacent to the capillary dropouts, intraretinal microvascular abnormalities are found as vascular shunts in severe NPDR. Preretinal collaterals can be detected in more advanced stages of NPDR. Vascular loops with larger caliber than that of neovascularization are observed in OCTA at the preretinal level, while

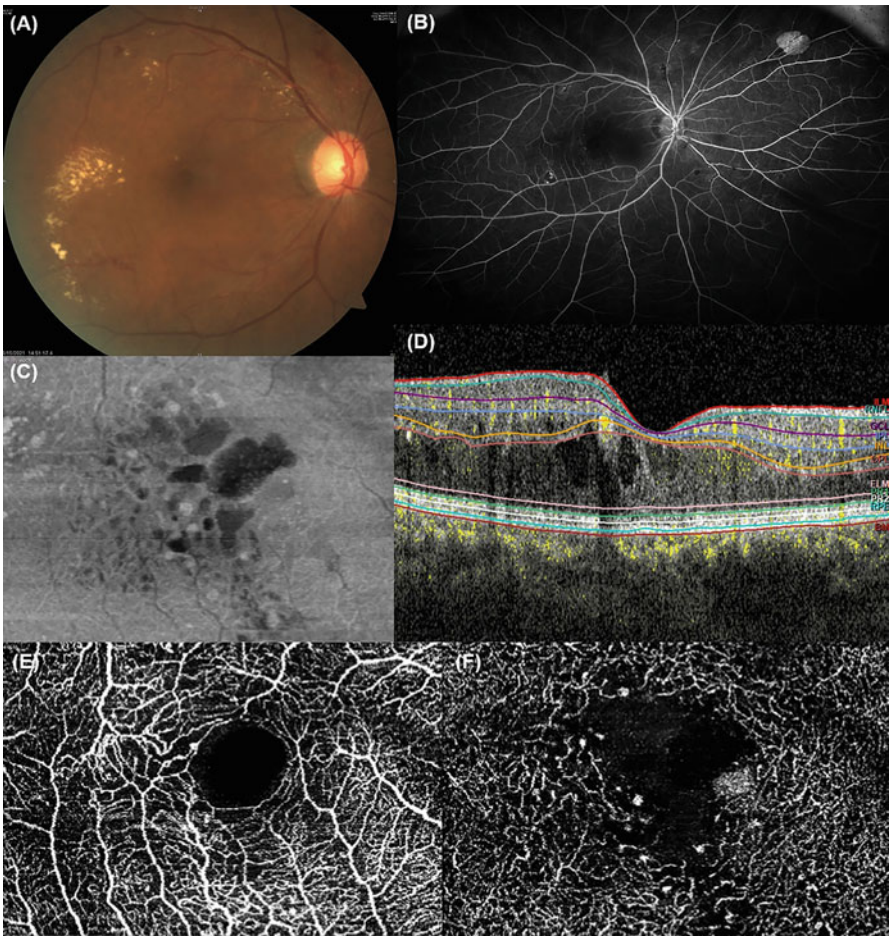

Fig. 2 Images of a patient with nonproliferative diabetic retinopathy and macular edema. (**a**) Color photography of microaneurysm hard exudates. (**b**) Early-frame fluorescein angiography (FFA) showing some microaneurysms around a local perfusion defect. Detailed changes in the foveal microvasculature are difficult to discriminate. (**c**) Near-infrared images showing low reflectance for cystoid edema and high reflectance for microaneurysms. (**d**) An 8.8-mm-wide horizontal B-scan with layer segmentations to indicate thickening due to leakage. (**e**) Enface optical coherence tomography angiography images (4.3 × 1.9 mm) of the superficial capillary plexus (**e**) and deep capillary plexus (**f**). Panel (**e**) presents enlargement of the foveal avascular zone with capillary loss; panel (**f**) shows impaired microvasculature around the fovea adjacent to some microaneurysms that are not visible on the FFA

they show no leakage in the late-phase FFA (Matsunaga et al. 2015). Decreased vascular density was observed on OCTA in the superficial and deep capillary plexus and choroidal capillary layer (Al-Sheikh et al. 2016; Bradley et al. 2016; Choi et al. 2020). The reduction in vascular density is more significant according to the severity of NPDR (Bhanushali et al. 2016). Therefore, quantification of vascular density by OCTA can be used as an objective biomarker of macular ischemia and DR severity.

Decreased vascular density in the deep capillary plexus is associated with thinning of the ganglion cell-inner plexiform layer, which reflects retinal neurodegeneration (Hafner et al. 2019). Choriocapillaris flow impairment was observed in DR and was correlated with DR severity and photoreceptor damage (Choi et al. 2017; Dodo et al. 2017). Reduced optic nerve head (ONH) vascularity was also reported as a change preceding retinal nerve fiber layer impairment in early-stage DR (Li et al. 2019). With the progression of DR, OCTA may reveal more pronounced choriocapillary ischemia (Li et al. 2019), which might lead to the disruption of the outer retinal photoreceptor layers (Dodo et al. 2017).

Proliferative Diabetic Retinopathy

PDR is defined as the presence of neovascularization and/or vitreous hemorrhage. Neovascularization is characterized by pathologic angiogenesis that grows from the retina into the vitreous and is found on the ONH or elsewhere adjacent to the non-perfusion areas (Matsunaga et al. 2015; de Carlo et al. 2016a). FFA can detect neovascularization, while detailed visualization of its morphology can be limited by fluorescein leakage. By using enface angiograms combined with B-scans, OCTA can reveal detailed morphological features along with the location and extent of neovascularization (Hwang et al. 2015) (Fig. 3).

Intravitreal anti-vascular endothelial growth factor (VEGF) injection and panretinal photocoagulation (PRP) are the mainstays of PDR treatment and act by reducing retinal neovascularization. OCTA is widely used to monitor changes in pathologic angiogenesis after treatment because it can demonstrate the capillary network without dye injection. A significant reduction in neovascularization was shown on OCTA within 2 weeks of anti-VEGF treatment, and a further decrease was observed in the area after 4 weeks (Ishibazawa et al. 2015). The remaining abnormal new vessels were reperfused and enlarged with irregular vasculature after 8 weeks. Similar changes in neovascularization were observed using OCTA 2 months after PRP treatment (Ishibazawa et al. 2016). OCTA can discriminate the location of neovascularization and help predict treatment response in advance. Neovascularization below the inner limiting membrane (ILM), which is more affected by retinal circulation, tends to regress with PRP treatment, whereas that above the ILM is more sensitive to anti-VEGF treatment (Chatziralli et al. 2016).

Diabetic Macular Edema

Diabetic macular edema (DME) is the most common cause of visual impairment in diabetic eyes, especially in NPDR (Dervenis et al. 2017). DME appears on OCT as oval blackout areas surrounded by abruptly stopping capillaries (Lee et al. 2016; de Carlo et al. 2016b), thus being morphologically different from non-perfusion areas. DME eyes have a lower vascular density, larger FAZ area, and more microaneurysms in the deep capillary plexus than non-DME eyes (Lee et al. 2016).

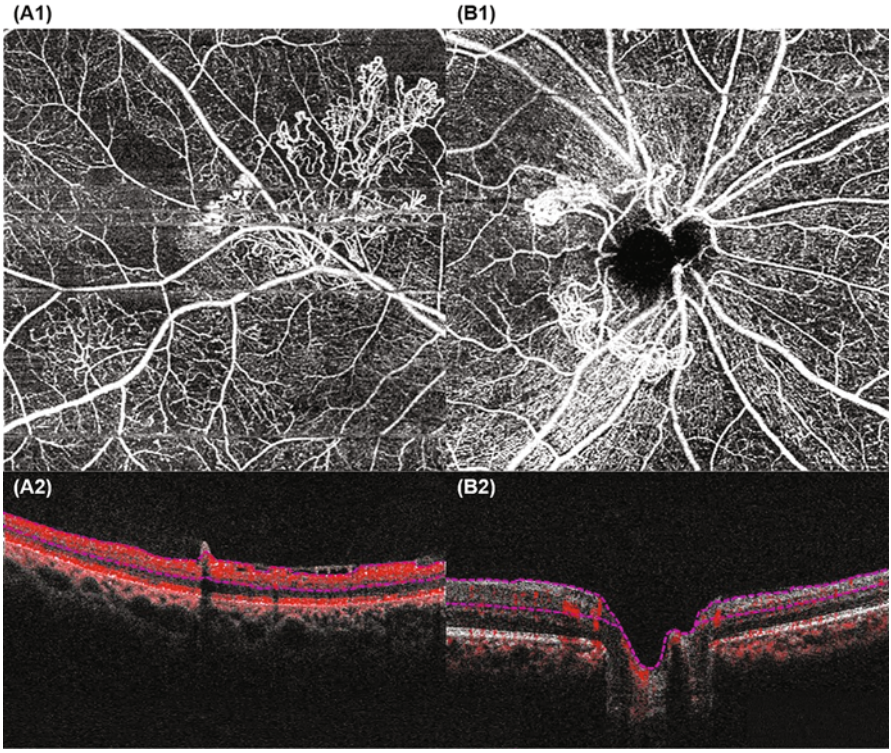

Fig. 3 Images of a patient with proliferative diabetic retinopathy with neovascularization. Enface optical coherence tomography angiography images (6 × 6 mm) of the superficial capillary plexus show (**a1**) the loop-like complex structures of neovascularization on the vascular arcade and (**b1**) neovascularization of the optic disc. Cross-sectional scans at the corresponding areas reveal the neovascularization lesion with blood flow projecting into the vitreous space (**a2** and **b2**)

After intravitreal anti-VEGF injections, which are one of the main treatments for DME, foveal cystoid spaces decrease, followed by replacement with retinal tissues (de Carlo et al. 2016b). The response to anti-VEGF treatment has been shown to be poorer in eyes with DME and lower vascular density, larger FAZ area, and more microaneurysms in the deep capillary plexus (Lee et al. 2016). Therefore, OCTA can be used to predict the response of DME to anti-VEGF agents.

Applications to Prognosis and Other Diseases or Conditions

Application to Diabetic Retinopathy Screening and Its Monitoring

The qualitative visualization by OCTA on microvascular changes following DR has improved our understanding of the pathophysiology of DR. Compared to FFA, OCTA provides higher contrast images of diabetic changes in the microvasculature

without the risk of images being obscured by fluorescein leakage. OCTA can also provide cross-sectional views that allow the detection of the vertical location of diabetic neovascularization. Since OCTA does not require administration of contrast dye, it can be repeated safely even in patients who are not indicated for FFA because of renal dysfunction.

Wide-field OCTA can improve the early detection of non-perfusion and neovascularization in the peripheral retina, while swept-source OCTA allows better visualization of the choriocapillaris by deeper signal penetration. In addition, OCTA enables quantitative measurements in each capillary plexus of the superficial and deep retinal and choroidal layers. This capability of OCTA is used as a tool for screening DR before it is clinically detectable and for monitoring DR progression. Therapeutic planning of each DR case by predicting treatment response is also a possible use of OCTA. It also emphasizes that the information on microvascular changes obtained through OCTA might serve as an important biomarker for retinal neurodegeneration as well as systemic diabetic control.

Application to Retinal Vascular Diseases

In addition to diabetic retinopathy, OCTA is also useful for retinal vascular diseases, especially for assessing perfusion changes and therapeutic effects. Retinal vein occlusion showed no significant improvement in vascular perfusion after anti-VEGF treatment (Sellam et al. 2017; Mastropasqua et al. 2017). Paracentesis in a case of retinal artery occlusion and anti-VEGF injection in a case of ocular ischemic syndrome showed dramatic improvement in retinal blood flow (Choi et al. 2018).

Limitations of OCTA

In addition to the abovementioned strengths, OCTA has some limitations. OCT artifacts can be caused by projection artifacts, segmentation errors, low signal strength, local signal loss, and eye movement (Spaide et al. 2018). The artifacts need to be considered for the correct interpretation of OCTA images. In contrast to FFA, OCTA cannot visualize differences in vascular permeability or changes in leakage. Quantitative information from OCTA mainly reflects the structure of the vascular network and not the actual state of blood flow. The comparison of acquired data from different OCTA instruments is also limited, as each result highly depends on the scan protocols, signal processing, and methods used to generate the OCTA images.

Mini-Dictionary of Terms

- **Optical coherence tomography angiography.** A noninvasive imaging technique that allows rapid generation of three-dimensional angiographic images of the retinal and choroidal vasculature.

- **Diabetic retinopathy.** A complication of diabetes caused by hyperglycemia-induced damage to the blood vessels in the retina and can lead to pathologic neovascularization.
- **Fovea avascular zone.** A specified region located at the center of the macula with the highest density of cone photoreceptors. It is devoid of retinal capillaries and is vascularized by the choroidal blood vessels.
- **Neovascularization.** Abnormal formation of new blood vessels, usually in the form of microvascular networks, to serve as collateral perfusion in response to local ischemia.
- **Vascular endothelial growth factor.** A signaling protein that promotes the growth of new blood vessels plays a central role in mediating microvascular and macrovascular pathology in diabetes.
- **Panretinal photocoagulation.** A type of laser treatment for the eye with newly developed abnormal blood vessels in the retina.

Key Facts of Optical Coherence Tomography Angiography (OCTA) in Diabetic Retinopathy (DR)

OCTA noninvasively visualizes the structure and flow of retinal vasculature layer by layer.

DR, a major microvascular complication of diabetes mellitus, causes various abnormalities in the retinal vasculature.

OCTA can be used as a screening tool for DR in patients with diabetes.

It can detect enlargement of the foveal avascular zone (FAZ) and decrease in vascular density of the deep capillary plexus before presentation of clinically significant retinopathy.

These changes become more pronounced with the progression of DR and are well correlated with serum biomarkers representing pancreatic β-cell function and insulin sensitivity.

Quantification of vascular density by OCTA can be used as an objective biomarker of macular ischemia and DR severity.

In the nonproliferative stage of DR, microaneurysms and intraretinal vascular shunts have higher contrast in OCTA than in fundus fluorescein angiography.

Detailed visualization of diabetic neovascularization is also enabled by OCTA in the proliferative stage.

OCTA can be safely repeated without the use of contrast media, making it suitable for monitoring the progression of DR and assessing the therapeutic response in patients with diabetes, especially those with renal dysfunction.

Summary Points

- DR has a significant impact on the retinal vasculature; thus, OCTA can have a wide range of applications in this field.

- Enlargement of the FAZ and decrease in the vascular density of the deep capillary plexus can be observed using OCTA prior to the onset of clinical retinopathy.
- OCTA can be used as a tool for screening DR before it is clinically detectable.
- These changes in the microvasculature can be used as biomarkers of pancreatic β-cell function and insulin sensitivity.
- Morphologic features and the extent of diabetic vascular abnormalities are well visualized by OCTA without the risk of images being obscured by dye leakage.
- Microaneurysms and intraretinal vascular shunts are observed in the non-proliferative stage, and neovascularization of the disc head and retina elsewhere is detected in the proliferative stage, which makes OCTA suitable for monitoring DR progression in patients with diabetes in whom the use of contrast media is contraindicated.
- OCTA can be used not only to evaluate but also to predict the therapeutic effect in DR cases.
- Microvascular changes can be quantified by OCTA and serve as an objective biomarker of macular ischemia and DR severity.
- However, the quantified data depend on the scanning equipment, protocols, and image processing methods.
- OCTA image interpretation should consider artifacts mainly caused by projection, segmentation errors, low signal strength, and eye movement.

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Urinary Profiling with Liquid Chromatography-Mass Spectrometry

38

Applications to Prediabetes in Animal Models

Lay-Harn Gam

Contents

Introduction 792
Urine as Diagnosis Specimen 793
Animal Model in Studying Human Disease 793
Liquid Chromatography-Mass Spectrometry 794
Application of Orthogonal Partial Least Squares Discriminant Analysis (OPLA-DA) to Prediabetes Diagnosis 795
Benefits of Physical Activity and Dietary Control 797
Proteomics Analysis of Urinary Proteins 799
Common Protein Biomarkers for Diagnosis of Diabetes 800
Application of Major Urinary Proteins and Histone H4 as Prediabetes Biomarker Proteins 801
Conclusion 803
Key Facts of Prediabetes 803
Mini-Dictionary Terms 803
Summary Points 804
References 804

Abstract

Prediabetes is a metabolic syndrome characterized by an elevated blood sugar. About 5–10% of prediabetes developed into diabetes. Prediabetes usually goes unnoticed as it is symptomless; however, it can be diagnosed by measuring the blood glucose levels, which is done invasively and therefore is not a favorable device by many. Noninvasive diagnosis tools by using urine specimens are desired. Urine contains useful biomarkers, namely, metabolites and proteins, that can be used to indicate the progression of prediabetes. Liquid chromatography separation coupled with mass spectrometry detector is useful in analyzing both small molecule metabolites and also macromolecule proteins. In addition,

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OPLS-DA patterns of the urinary profiles of metabolites may also be used as indicator for prediabetes. This approach can be used to evaluate the state of disease by using urine specimen; furthermore, the impact of diet on the state of prediabetes disease can also be evaluated.

Keywords

Prediabetes · Animal study · Urinary metabolites · Urinary proteins · Mass spectrometry analysis

Abbreviations

ESI	Electrospray ionization
FBG	Fasting blood glucose
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
HFD	High-fat diet
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MUPs	Major urinary proteins
NA	Nicotinamide
ND	Normal diet
OPLS-DA	Orthogonal partial least squares discriminant analysis
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus

Introduction

The Centers for Disease Control and Prevention (US) reported that prediabetes affects 37% and 29% of male and female, respectively, of aged >18 years. Prediabetes is a component of metabolic syndromes characterized by elevated blood sugar, although not high enough to be grouped as diabetes. The cause of prediabetes is related closely to genetic factor and lifestyle. Prediabetes case is on the rise, indicating that lifestyle is the main factor contributing to the state of prediabetes. About 5–10% of prediabetes developed into diabetes; therefore, active physical activity and control dietary intake are necessary to prevent the progression of the disease. People with prediabetes can live with it for years without noticing it; this is because there's no symptom to indicate the condition. In general, people will aware that they are prediabetes through routine blood testing; nevertheless, routine blood testing is not a routine practice for many. This is because it is an invasive testing, making it not favorable by many. In view of this, urine will provide a good source of testing; therefore, a good urinary testing device will be desired to achieve this goal.

Urine as Diagnosis Specimen

Urine is a transparent, sterile, and yellowish-colored fluid produced by mammals. Urine is the primary route to eliminate water-soluble waste products such as sugars, urea, inorganic salts, organic acids, creatinine, ammonia, various toxins, and products of metabolism breakdown (Bouatra et al. 2013). Urine can be collected noninvasively and longitudinally (Wald 2017). Furthermore, urine lacks homeostasis mechanism (Gao 2013); therefore, it is a rich bio-fluid for medical diagnostic (Khamis et al. 2017). There are approximately 4500 metabolites detected in urine, associated with close to 600 human conditions such as obesity, diabetes, cancer, inflammation, etc. (Bouatra et al. 2013; Khamis et al. 2017; Miller et al. 2019). Besides diseases, intrinsic factors (age, gender, and hormonal status) and extrinsic factors (lifestyle, diet, and exercise) will affect the composition of urinary metabolites (Wu and Gao 2015). Dietary intake can affect kidney filtration and tubular reabsorption by interfering with the acid-base balance, water levels, electrolyte, and various metabolisms in the body. Urine provides a more sensitivity indicator on changes in diet compared to blood or saliva (Walsh et al. 2006). Therefore, urine has been used in discovery of biomarkers related to dietary exposure or food intervention through urinary metabolomics studies (Gibbons et al. 2015; May et al. 2013; Pujos-Guillot et al. 2013). On the other hand, urinary proteins are composed of ~70% proteins that are originated from kidney and urinary tract, while 30% of the urinary proteins are originated from blood plasma (Xiao et al. 2019). Urinary protein is less complex compared to plasma protein although valuable proteins, peptides, and amino acids (Xiao et al. 2019) can be identified from urinary proteins that serve as valuable biomarkers for diagnosis tools (Gao 2013). Urinary biomarkers have been used as early diagnosis and prognosis of various diseases, including diabetes, cancer (skin, brain, lung, liver, and bone cavity), neurodegenerative diseases, respiratory diseases, renal diseases, as well as diseases related to the liver and pancreas (Gao 2019).

Animal Model in Studying Human Disease

Rats are the most widely utilized models in biomedical research. This is due to their genetic and physiological similarity to humans (Worp et al. 2010). Type 2 diabetes mellitus (T2DM) in the animal model could be induced via a combination of streptozotocin (STZ) and nicotinamide (NA) (Masiello et al. 1998). STZ mainly targets pancreatic β-cells and is transported into β-cells through the glucose transporter 2 (GLUT2) (Wu and Yan 2015). The exposure of β-cells to STZ results in DNA damage and causes increased activity of poly(ADP-ribose) polymerase to repair DNA. However, excessive activity of this enzyme leads to β-cell necrosis (Szkudelski 2012). STZ indicates destruction of Langerhans islet cells irreversibly and induces diabetes in rats. Nicotinamide could protect β-cells by inhibiting the poly(ADP-ribose) activity (Masiello et al. 1998). Hence, an optimal dosage of STZ and NA can be used to induce T2DM in animals. NA could partially protect β-cells

against the β-cytotoxic effect of STZ (Szkudelski 2012). Through this process, some of the rats formed T2DM; however, some of these induced rats are presented with a condition of prediabetes; according to the definition of its fasting blood glucose (FBG) levels, when FBG was >7 mmol/L, it is diabetes, but when FBC was between 5.6 and 6.9 mmol/L, this indicates the state of prediabetes. Previous study stated that STZ-induced diabetic rats produce more urine compared to the normal rats (Akbarzadeh et al. 2007). This phenomenon is called polyuria. Polyuria occurs when the kidney fails to respond to antidiuretic hormone, arginine vasopressin, resulting in excretion of a large amount of diluted urine (approximately 40 ml/kg/24 h) (Kavanagh and Uy 2019). In general, volume of urine collected in 24 h from diabetic rats was much higher compared to that of control rats and prediabetes rats.

Liquid Chromatography-Mass Spectrometry

Liquid chromatography coupled with mass spectrometry detector is widely used in analysis of metabolomics and proteomics. The LC-MS instruments combined protocols of liquid chromatography and mass spectrometry in separating and analyzing analytes. Reversed-phase LC columns are usually used in chromatography for separation of the mixture of analytes (Milac et al. 2012). In proteomics analysis, nano LC has gain popularity because of its enhanced sensitivity (Gama et al. 2013). Nano LC with low i.d. (0.1 mm or lower) has a lower degree of band dilution and is able to concentrate samples before entering MS (Wilson et al. 2015). In urinary metabolomics analysis, an analytical column (4.6 mm i.d.) is usually sufficient, provided that the volume of urine is not a limiting factor. An LC-MS/MS instrument is made up of an ionization source, an ion-inlet and focusing component, a first mass-filtering device, a collision chamber, a second mass-filtering device, and an ion-impact detector (Grebe and Singh 2011). An ionization source is needed to prepare the analytes into charged gas particles before entering MS. In electrospray ionization (ESI), high voltage around 2–6 kV is applied allowing dispersion of sample solution into highly charged droplets, whereby these droplets will progressively desolvate into gaseous ions before entering to MS; this is done with the aid of drying gas and heated capillary (Banerjee and Mazumdar 2012). The general principle of tandem MS involves the isolation of precursor ions in the first mass analyzer and fragments them in a collision chamber to yield product ions which will be analyzed by the second mass analyzer (Madeira and Florêncio 2012). MS/MS analysis provides the spectra of the fragment ions of a precursor ion, which can be a peptide in proteomic analysis. These spectra which are associated with the fragmentation of the peptide can be used to calculate the amino acid sequence of the peptide and thereafter give identification to the protein due to the unique amino acid sequence of each protein. This approach has been used in protein identification (Soares et al. 2012). The downstream analysis of the data obtained to identify the proteins can be done by using software and database search engines, for example, MASCOT, X!Tandem, MassQuant, PeaksDB, and MS-GF+ (Verheggen et al. 2020). On the other hand, metabolomics analysis is usually sufficient by using LC-MS. The

base peak chromatogram profiles can be used for identification of the disease condition by subjecting them to statistical analysis tools such as OPLS-DA. Nevertheless, standard markers may be needed for the purpose of identification of the unknown markers.

Application of Orthogonal Partial Least Squares Discriminant Analysis (OPLA-DA) to Prediabetes Diagnosis

Numerous metabolites such as lysophosphatidylcholines (Urpi-Sarda et al. 2015), phenylalanine (Tam et al. 2017), 5′-methylthioadenosine (Zhang et al. 2016), cyclic adenosine monophosphate (Zhang et al. 2016), and acetylhistidine (Zhang et al. 2016) have been detected in both urine and blood at different concentrations and were identified as biomarkers for the occurrence and risk development of T2DM and prediabetes in both human and animal models (Sas et al. 2015). Besides the use of specific biomarkers as indicators, the profiling of mass chromatogram of urine as a whole can also be used as indicators for the state of disease.

Orthogonal partial least squares discriminant analysis (OPLS-DA) has been widely used in the analysis of omics data that are untargeted (Wang et al. 2019). It is most useful when the generated urinary profiles cannot be singularly used to differentiate the disease condition. In an experimental designed, it is hoped that the treatment effect is equal for all subjects; nevertheless, within treatment variation depicts the average effect of treatment leads to systematic remainder variation, which is not related to the treatment (Wiklund et al. 2008), causing challenges in extracting knowledge from these data. In this situation, the urinary metabolite profiles can be used to generate OPLS-DA score, from which the control, diabetic, and pre-diabetic rats can be differentiated. Urine is a better source of liquid specimen because urine collection can be done noninvasively and therefore reduces the risk of infection (Miller and Peters 2019). Furthermore, urine contains many stable metabolic end products and fewer protein complexes and lipids, while blood is highly dynamic, and blood sample handling can introduce pronounced changes to the metabolome (Emwas et al. 2015). Therefore, sampling of urine is preferable, as it will be safer, have better reproducibility, and be noninvasive.

The progression of diabetes has been shown to alter the urinary metabolite profile which led to the identification of relevant biomarkers (Tam et al. 2017; Guan et al. 2013). In a previous study conducted in the author's laboratory, LC-MS analysis of urine coupled with OPLS-DA statistical analysis tool has been shown to give unique patterns of OPLS-DA that separate healthy, prediabetes, and diabetes conditions.

A three-dimensional comparison between urinary OPLS-DA of control rats, pre-diabetes rats, and diabetes rats was carried out (Lee et al. 2020). In general, less separation between the two intended groups indicates closer similarity in the metabolic states. Such analysis has shown that the metabolic state of pre-diabetic rats is more similar to control rats and that a clear metabolic shift upon progression to full diabetes was observed (Fig. 1). A separation was observed between the control and prediabetes rats fed on the same healthy diet, indicating different biochemical processes between

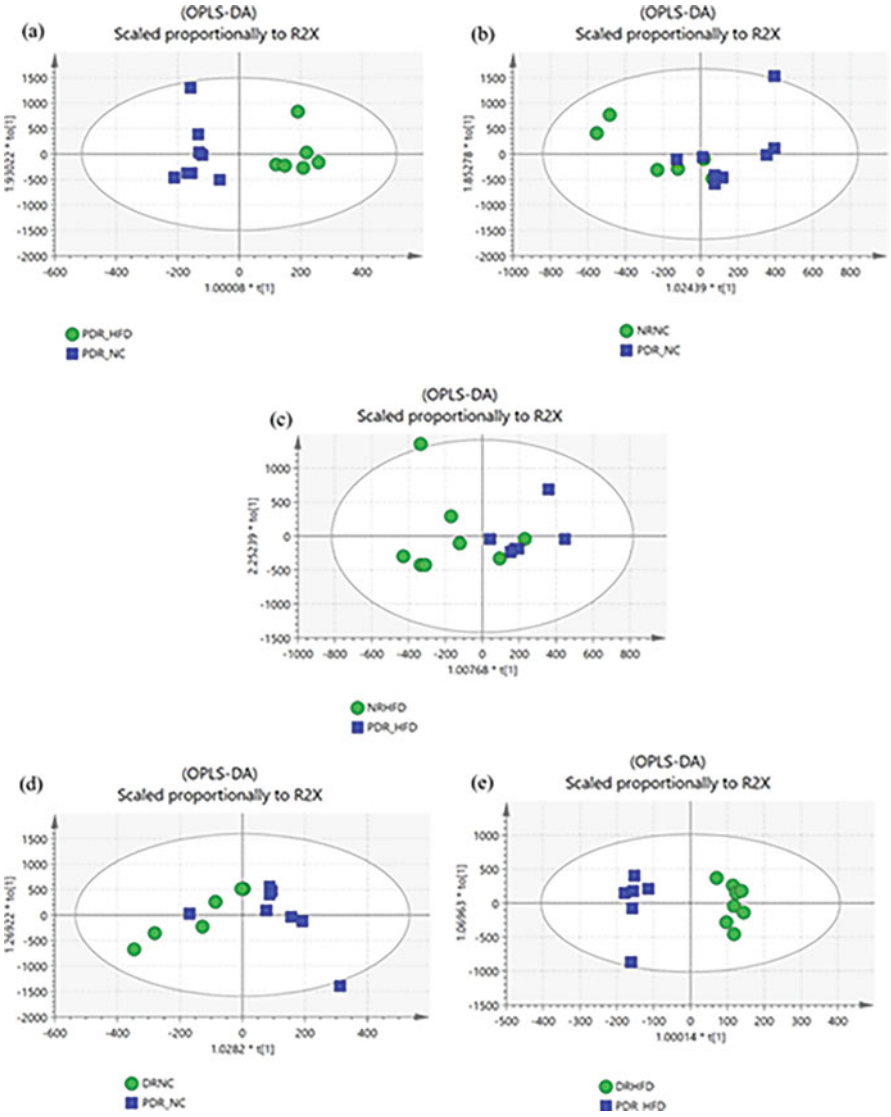

Fig. 1 Orthogonal partial least squares discriminant analysis (OPLS-DA) score scatterplots of control, diabetic, and pre-diabetic urine samples derived from data collected from UPLC/ESI-QTOF-MS operated in positive ionization mode. NR, control (non-induced); DR, induced diabetic; PDR, pre-diabetic; NC, fed with normal chow diet; HFD, fed with high-fat diet. (Figure obtained from Lee et al. 2020, The International Union of Biochemistry and Molecular Biology and Wiley-Blackwell Publisher)

normal and prediabetes rats that led to differences in urinary metabolite profiles. OPLS-DA for pre-diabetic rats with healthy diet responded to metformin as less separation was observed when compared to control rats fed with healthy diet. The

less separation means the higher similarity between the two groups of comparison. This indicates an improved form of the disease. Furthermore, metformin treatment has been shown to benefiting the prediabetes condition as a clear separation between prediabetes group and prediabetes groups treated with metformin was detected (Fig. 2), this is supported by the FBG levels where upon metformin treatment, the FBG of prediabetes rats were reduced to the same levels as control rats. In pre-diabetic rats fed with healthy diet, metformin helps to normalize the urinary metabolite profile toward the pattern seen as control rats. On the contrary, in pre-diabetic rats fed with high-fat diet, a clear separation from normal rats remained even after treated with metformin indicating prediabetes condition is not improved with metformin treatment if high-fat diet is consumed daily. The effect of high-fat diet on prediabetes rats was more profound than the healthy diet, where a distinct separation was observed between the prediabetes rats and control rats fed with high-fat diet when compared with the corresponding groups fed with healthy diet.

Prediabetes marks the early commencement of T2DM; we found that the OPLS-DA pattern separates between healthy diet and high-fat diet in prediabetes, and the latter is more similar to that of diabetics. In addition, unlike diabetes rats, prediabetes rats fed with NC responded to metformin treatment. Therefore, using the combination of urinary metabolite analysis by LC-MS and OPLS-DA, profile patterns for healthy, diabetes, and prediabetes not only can be used as a diagnostic tool to diagnose prediabetes condition. Furthermore, the shift of OPLS-DA score has been shown proportional to the impact of diet and lead to progression or regression of prediabetes condition, where, if proper care and precaution are given at prediabetes stage, diabetes can be avoided. Therefore, this approach can be used as a reference for the prevention of progression of prediabetes to T2DM.

Benefits of Physical Activity and Dietary Control

Physical activity along with dietary control is beneficial in managing blood glucose and maintaining good health in people living with prediabetes (Colberg et al. 2016). Most glucose uptake occurs in the skeletal muscle, and the translocation of glucose transporter (GLUT) carrier proteins, particularly GLUT4, is important in regulating glucose transport. Its level determines insulin sensitivity (Hughes et al. 1993). At rest, glucose uptake is insulin-dependent, while exercise stimulates the muscles to utilize the glucose, which is made available by intramuscular glycogenesis and therefore increases glucose uptake (Colberg et al. 2010). Both aerobic and resistance (strength) exercises have been linked to improved blood glucose control by increasing the glucose uptake in muscle via insulin-independent mechanisms that increase GLUT4 abundance and translocation. After exercise, glucose uptake into contracting muscle remains elevated and active for a few hours (Goodyear and Kahn 1998). Hence, regular aerobic and resistance exercise improves insulin sensitivity and insulin action in muscle tissue of prediabetes patients. Physical exercise also strengthens the endurance of cells, tissues, and organs to oxidative stress and increases energy metabolism, vascularization, and neurotrophic synthesis (Chen

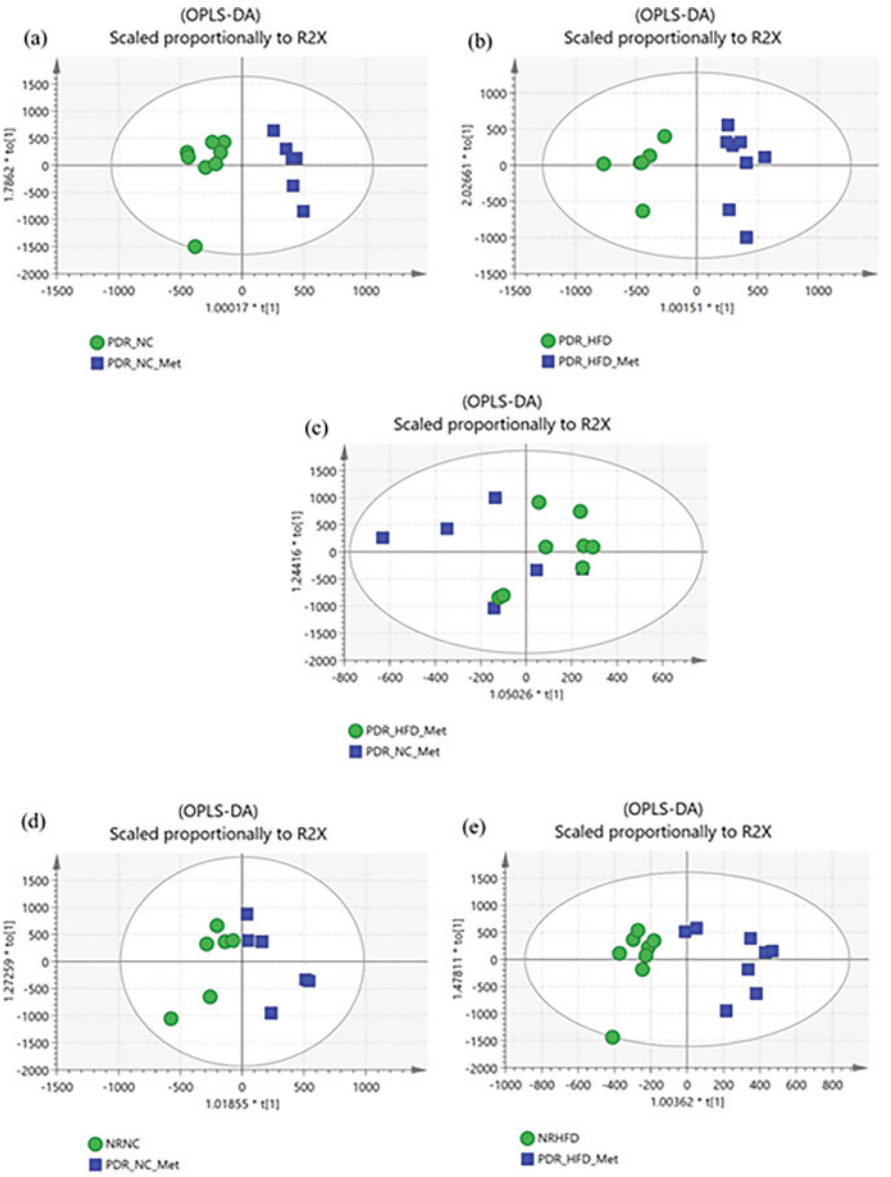

Fig. 2 Orthogonal partial least squares discriminant analysis (OPLS-DA) score scatterplots of pre-diabetic rat models treated with metformin. NR, control (non-induced); DR, induced diabetic; PDR, pre-diabetic; NC, fed with normal chow diet; HFD, fed with high-fat diet. (Figure obtained from Lee et al. 2020, The International Union of Biochemistry and Molecular Biology and Wiley-Blackwell Publisher)

et al. 2016). People living with diabetes and prediabetes should regularly exercise to prevent progression of complications including Alzheimer's disease and cardiovascular and kidney diseases. In conclusion, metformin could potentially be used together with a healthy diet and regular exercise to help prevent the progression of prediabetes into diabetes.

Proteomics Analysis of Urinary Proteins

Urine can also be subjected to urinary protein analysis; it is a useful approach for identification of protein biomarkers for diagnosis or monitoring the progression of disease condition, namely, the progression of prediabetes to diabetes. Protein is the functional component of cell; therefore, protein not only can serve as a diagnosis biomarker; the identity or function of unique or differential excretion of urinary proteins between disease and healthy conditions can also indicate cellular disorder caused by the disease state. Proteins are present at low quantity in urine; therefore, concentrating the purification of urine is needed before analysis. Sample preparation can be done by salt precipitation using ammonium sulfate. Ammonium sulfate salt is relatively inexpensive and can prevent denaturation of proteins (Burgess 2009). Increasing salt saturation leads to increment of water surface tension as well as hydrophobic interaction between protein and water, which caused solubility of protein to decrease and then promote the aggregation of proteins (Wingfield 1998). However, solubility of proteins varies as a function of ammonium sulfate concentration (Burgess 2009). In the author laboratory, 45% salt saturation was found to give the best urinary protein yield.

Urinary protein mixture is not complex as compared to protein mixtures obtained from cells or tissues. Therefore, good urinary protein separation can sufficiently be achieved by one-dimensional gel electrophoresis, where the proteins are separated into discrete fractions of protein bands. Methods such as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and tricine gel electrophoresis can be used to achieve this purpose. SDS-PAGE is a simple yet reliable method to resolve proteins by molecular weights. Glycine SDS-PAGE is commonly used to resolve proteins above 10 kDa, while tricine SDS-PAGE is used for resolving the lower molecular weight peptides. This is due to the stacking limit in stacking gel of tricine shifts to a low molecular mass range when glycine is substituted with tricine, whereby tricine migrates much faster than glycine in stacking gel due to its higher pKa value (Schägger and Von Jagow 1987). The unique or differential excretion protein bands can be identified by comparison of the protein profiles of control and prediabetes urine; these protein bands can be excised individually and subjected to in-gel digestion using trypsin, and the resulted peptides can be analyzed using LC-MS/MS method as described earlier. The MS/MS spectrum can be subjected to protein database search, from which the identity of the protein is made known.

Common Protein Biomarkers for Diagnosis of Diabetes

The classical biomarkers used for clinical diagnosis of diabetes included glycated hemoglobulin (HbA1c) and glycated albumin (GA). The level of HbA1c reflects the average plasma glucose for a period of ~2–3 months; therefore, using HbA1c was said to be a better reference value than fasting glucose level, which only represents blood glucose level at a single time point (Vaishya et al. 2018). Moreover, HbA1c has a greater pre-analytical stability, and it is less affected by perturbation (Dorcely et al. 2017). Nevertheless, the levels of HbA1c are affected by red blood cell titer; reduced red blood cell turnover associated with iron, vitamin B12, and folate deficiency anemia can cause false elevation of HbA1c (Lee 2015). HbA1c was first introduced by ADA in 2009, and it was widely used for diagnosis of T2DM since then with thresholds of HbA1c $\geq$ 6.5% (American Diabetes Association 2019).

Another biomarker that was used to index glycemic control is glycated albumin (GA). GA is defined as the ratio of serum GA to total albumin. GA has greater glycation speed than HbA1c although it has a shorter life span than the latter; hence, it is used to measure a shorter duration of glycemic control (within 2–3 weeks) (Lee 2015). GA levels is interfered by liver cirrhosis and hypothyroidism; furthermore, low GA levels were also observed in people with increased BMI, body fat mass, visceral adiposity, and obesity (Dorcely et al. 2017). All these reasons make the use of this classical biomarker inconclusive for prediction of T2DM progression (Vaishya et al. 2018).

Urine is formed from blood plasma by ultrafiltration process in the kidney and then excreted through the urinary tract. Thus, the urinary proteome may reflect the functions of the related organ, namely, the kidney. There are multiple reports on the potential urinary biomarkers that can be used to predict complications related to diabetes, particularly diabetic nephropathy (DN). Increased excretion of Ig kappa light chain, transthyretin, and plasma retinol-binding protein has been associated with the impairment of kidney tubular reabsorption function in patients with diabetes (normoalbuminuric) (Bellei et al. 2008). Elevated levels of albumin, retinol binding protein 4, zinc-α2-glycoprotein, and E-cadherin and downregulation of bikunin precursor and haptoglobin precursor were observed in the progression of diabetes to diabetic nephropathy (Riaz et al. 2010). Urinary biomarkers such as transferrin, type IV collagen, N-acetyl-β-D-glucosaminidase, ceruloplasmin, free light chains, and kidney injury molecule 1 were reported in the progression of renal dysfunction (Currie et al. 2014; Matheson et al. 2010). Urinary biomarkers that reflect inflammatory response, namely, monocyte chemoattractant protein-1, tumor necrosis factor-α, vascular endothelial growth factor and transforming growth factor-β, and a few oxidative stress biomarkers, which include 8-hydroxy-2′deoxyguanosine, α-1-acid glycoprotein, and pentosidine, have been proposed to be used in the monitoring of diabetes-related kidney disease (Currie et al. 2014; Matheson et al. 2010). Moreover, peptides, such as histidine triad nucleotide-binding protein, bifunctional aminoacyl-tRNA synthetase, and clusterin precursor protein, have been identified in the urine of diabetes patients (Chu et al. 2013). Biomarker for diabetic nephropathy,

CKD273, was said to be useful in determining the prognosis of diabetic nephropathy (Klein et al. 2016). The increased excretion of several urinary proteins or peptides, namely, transthyretin, Ig kappa chain C region, cystatin C, and ubiquitin, can be the early signs of tubular damage, before macroalbuminuria is established (Patel and Kalia 2019). All of these identified urinary biomarkers are advantageous as detection tools for T2DM and associated renal complications.

Application of Major Urinary Proteins and Histone H4 as Prediabetes Biomarker Proteins

In the author's laboratory, tricine-PAGE urinary protein profiles for healthy, prediabetes, and diabetes rats were analyzed (Teh et al. 2020a, b). Figure 3 shows the gel profile of control and diabetes rats, while Fig. 4 shows the gel profile of control and prediabetes rats. As can be seen in Fig. 4, control rats and prediabetes rats shared many similarities, while the diabetes rats have quite a different profile from the control and prediabetes rats; attention can be drawn to band 10, a 15.9 kDa peptide; band 11, a 14.9 kDa peptide; and band 13, a 9.8 kDa peptide. These three protein bands were consistently detected in healthy and prediabetes rats, while they were not detected in diabetes rats. The excretion of the three bands in the animals was not

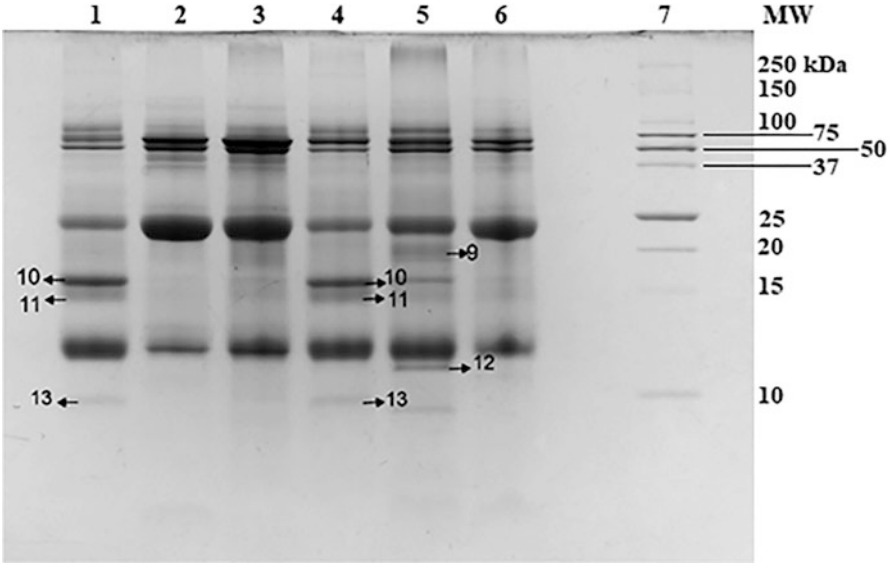

Fig. 3 Tricine SDS-PAGE of rat urinary proteins from diabetic groups and control groups. Lane 1, nondiabetic rat fed with ND (G1); Lane 2, diabetic rat fed with ND (G3); Lane 3, diabetic rat fed with ND with metformin treatment (G5); Lane 4, nondiabetic rat fed with HFD (G2); Lane 5, diabetic rat fed with HFD (G4); Lane 6, diabetic rat fed with HFD with metformin treatment (G6); Lane 7, protein ladder. (Figure obtained from Teh et al. 2020a Journal of Scientific and Technical Research)

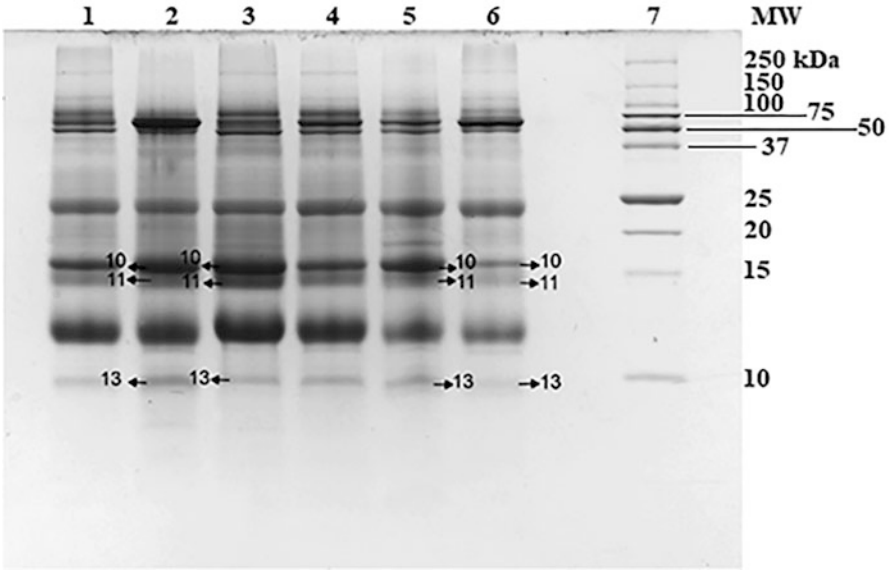

Fig. 4 Tricine SDS-PAGE of rat urinary proteins from pre-diabetic groups and control groups. Lane 1, nondiabetic rat fed with ND (G1); Lane 2, Pre-diabetic rat fed with ND (G7); Lane 3, pre-diabetic rat fed with ND with metformin treatment (G9); Lane 4, nondiabetic rat fed with HFD (G2); Lane 5, pre-diabetic rat fed with HFD (G8); Lane 6, pre-diabetic rat fed with HFD with metformin treatment (G10). (Figure obtained from Teh et al. 2020a Journal of Scientific and Technical Research)

Table 1 Identified protein list. (Table obtained from Teh et al. 2020a Journal of Scientific and Technical Research)

Band label	Protein	Accession	-10logP	MW (kDa)
10	Major urinary protein	P02761	94.26	15.9
11	Major urinary protein	P02761	95.78	14.9
13	Histone H4	P62806	89.85	9.8

affected by the diet intakes (healthy or high-fat diet) and treatment with metformin. Therefore, these three bands can be used as biomarkers to monitor the progression of prediabetes to diabetes state. The similarity of healthy and prediabetes rats' urine profiles also indicated a closer resemblance of these two groups of animals physiologically compared to the diabetes animals. The three bands were excised and digested in-gel using trypsin; the LC-MS/MS analysis indicated that both bands 10 and 11 were major urinary proteins while band 13 was histone H4 protein (Table 1).

Major urinary proteins (MUPs) are produced in the liver, transported to the kidney through the bloodstream, and excreted in urine. Circulating MUPs involve in glucose metabolism by suppressing hepatic gluconeogenesis as well as improving energy expenditure and insulin sensitivity in skeletal muscles (Zhou and Rui 2010). In this study, MUPs were found in normal rats and prediabetes rat, but not found in

diabetes rats. The study has reported that insulin plays a regulatory role in hepatic synthesis of MUPs and insulin deficiency might be the reason of the suppression of this protein (Roy et al. 1980). Moreover, several proteomics studies on diabetic rats also identified a downregulated expression of MUPs in urine, kidney, and renal mitochondria (Sharma and Tikoo 2014).

Histone H4 is one of the core structural units of nucleosomes which take parts in regulating gene expressions. Histones are small positively charged proteins. This characteristic encourages their excretion into the urine; this is because histones can pass through the kidney's Bowman's space from the bloodstream (Kawai et al. 2016). The suppression of histone H4 in diabetes rats' urine as compared to the normal and prediabetes rats has not been reported previously. However, studies on epigenetic mechanisms of diabetes have suggested that the pathogenesis of diabetes is related to histone posttranslational modifications, which influence genetic expression of this protein in the organs associated with diabetic complications (Kato and Natarajan 2014; Reddy et al. 2013; Sayyed et al. 2010). Hence, the direct mechanism causing the suppression of histone in diabetic rat urine needs further investigations.

Conclusion

Recently, diagnosis of progression of prediabetes to diabetes is carried out invasively, where blood glucose level is measured. In author's laboratory, the use of urine for diagnosis purpose has been demonstrated. Metabolite profiles of urine by LC-MS analysis coupled with OPLS-DA or using of reliable urinary protein biomarkers may be an alternative way for diagnosis of diabetic in a friendlier and noninvasive environment. It may not be able to totally replace the current diagnosis test by using blood glucose; nevertheless, it can be used as a screening method for early detection of prediabetes condition and also for monitoring the progression of prediabetes to diabetes, especially among the elderly and in the diabetic-epidemic areas.

Key Facts of Prediabetes

- Prediabetes affects 37% and 29% of male and female.
- About 5–10% of prediabetes developed into diabetes.
- Prediabetes is related closely to genetic factor and lifestyle.
- Prediabetes case is on the rise.
- Lifestyle is the main factor contributing to the state of prediabetes.

Mini-Dictionary Terms

- **Prediabetes:** A metabolic syndrome demonstrated by elevated fasting blood glucose. A precondition of diabetes and it can progress into diabetes.

- **Metabolites urinary profile:** Base peak chromatogram of liquid chromatography-mass spectrometry analysis of metabolites in urine.
- **Urinary protein profile:** The pattern of urinary proteins as separated according to their respective molecular weights by using gel electrophoresis technique.
- **Orthogonal partial least squares discriminant analysis (OPLS-DA):** It is a useful statistical analysis device to generate usable information from a challenging and non-systematic data.
- **Biomarker:** An indicator obtained from human specimens that can reliably use to indicate the state of disease.

Summary Points

- Prediabetes is on the rise mainly due to lifestyle of individuals.
- A noninvasive method by using urine specimen is highly desired to provide early diagnosis of prediabetes before the progression of the disease.
- Separation and analysis techniques, namely, liquid chromatography and gel electrophoresis coupled with mass spectrometric analysis, provided good platforms in analysis of biomarkers.
- Urinary metabolite profiles by LC-MS analysis when coupled with OPLS-DA generate a distinct pattern for diagnosis of prediabetes and also its progression into diabetes.
- Urinary protein biomarker proteins can be identified from unique or differentially excretion of proteins, which can be used to indicate the state and progression of prediabetes.

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Physiologic Measures in Diabetes: QTc Prolongation

39

Lauren Ehrhardt-Humbert and Matthew J. Singleton

Contents

Introduction 810
ECG and the Myocardial Action Potential 810
QT Interval 811
Genetics of the QT Interval 812
Early ECG Changes in Diabetes 812
Glycemic Variability and the QT Interval 812
QT Interval in Diabetes Mellitus 814
QT Interval and Sudden Cardiac Death 814
QT Interval and Atrial Fibrillation 814
QT Dispersion 815
Pathologic Q Waves: Diabetic Risk of Silent Myocardial Infarction 815
REGARDS Study 815
Applications to Prognosis and Other Diseases or Conditions 816
Mini-Dictionary of Terms 816
Key Facts of QT Interval 816
Key Facts of Atrial Fibrillation 817
Key Facts of Malignant Arrhythmias 817
Key Facts of Sudden Cardiac Death 817
Summary Points 818
References 818

Abstract

Cardiovascular disease is the leading cause of mortality in diabetes, necessitating biomarkers beyond traditional methods to stratify risk in these populations. Glycemic variability and prolonged hyperglycemia have been linked to diabetic

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complications and increased risk of mortality. Early in the disease process, pathologic changes to the heart occur and can be captured with noninvasive methods including an electrocardiogram (ECG). Prolonged repolarization of cardiac myocytes, represented by a prolonged QT interval on ECG, predisposes the heart to arrhythmias and sudden cardiac death. Notably, variations in the QT interval such as prolonged QT interval and increased QT dispersion have been linked to increased all-cause mortality in those with diabetes.

Keywords

Diabetes mellitus · Hyperglycemia · QT interval · QT prolongation · Long QT syndrome · Short QT syndrome · QT dispersion · All-cause mortality · Sudden cardiac death · Malignant arrhythmia · Silent myocardial infarction · Atrial fibrillation

Abbreviations

AV node	Atrioventricular node
CPR	Cardiopulmonary resuscitation
DM	Diabetes mellitus
ECG	Electrocardiogram
QTc	Corrected QT interval
SA node	Sinoatrial node
SCD	Sudden cardiac death

Introduction

Diabetes mellitus (DM) is a chronic systemic disease with many comorbidities including renal and cardiovascular diseases. Cardiovascular disease, the leading cause of mortality in patients with diabetes mellitus, is complex and treatable. The Atherosclerosis Risk in Communities (ARIC) study found that those with undiagnosed diabetes, pre-diabetes, and diagnosed diabetes had a 1.3 and 3.1 times higher rate of hospitalization. Cardiovascular causes were one of the highest excess rates of hospitalization (Schneider et al. 2016). Thus, biomarkers of cardiovascular risk beyond traditional measures are needed. A routine 12-lead electrocardiogram (ECG) is a cost-effective method to identity early cardiac disease and stratify cardiovascular risk (Mould et al. 2021; Singleton et al. 2021b). This chapter will discuss the QT interval and the role of QTc prolongation in predicting all-cause mortality in diabetes.

ECG and the Myocardial Action Potential

Cardiac myocytes undergo electrical-mechanical coupling, a phenomenon in which myocytes contract in a synchronous manner in response to electrical action potentials in pacemaker cells. The action potential of cardiac myocytes is characterized into five

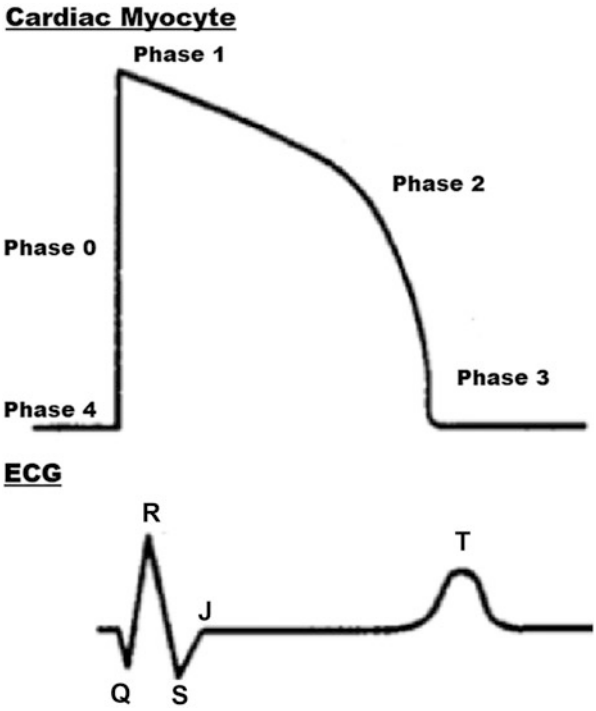

Fig. 1 Cardiac myocyte action potential and ECG wave form. This figure shows the phases of the ventricular cardiac myocyte action potential outlined in section "ECG and the Myocardial Action Potential." Each phase of the action potential corresponds to a particular duration of the P-QRS-T waveform recorded on the ECG. The QT interval corresponds to phase 0 through phase 3, reflecting depolarization, isovolumetric contraction, and repolarization of the ventricles (original image)

phases that correspond to movement of ions and a change in voltage (Priest and Mcdermott 2015). Phase 0 begins when inward voltage-gated sodium channels open causing a rapid upstroke and depolarization of the cell. Phase 1, the initial repolarization, occurs when those voltage-gated sodium channels are inactivated and voltage-gated potassium channels open. Slow calcium influx through voltage-gated channels balances out potassium efflux to create a plateau in Phase 2. Calcium inside the myocyte triggers release of calcium from the sarcoplasmic reticulum which facilitates actin and myosin interaction for contraction. Phase 3 starts as the calcium channels close, voltage-gated slow delayed rectifier potassium channels open, and a massive efflux of potassium ions occurs. Phase 4 completes the cycle as an effective refractory period dominated by potassium channels (Fig. 1). Each of the phases correlates to amplitude changes on the ECG (Bednar et al. 2001; Priest and Mcdermott 2015).

QT Interval

The QT interval correlates to the duration of the depolarization, mechanical contraction, and repolarization of the ventricles (Parts and Were 2009). It consists of a QRS complex, which measures His-Purkinje fiber depolarization, and the JT interval which measures ventricular repolarization. The T wave reflects serial repolarization of the

myocardial layers (epicardium and endocardium) from the apex of the heart to the base of the ventricles (Bednar et al. 2001; Priest and Mcdermott 2015). The QT interval is often reported as "QTc," or the QT interval corrected for heart rate. Normal values for the QT interval vary by genetics, gender, age, and comorbidities. In a review by Viskin, QTc interval is typically below 440 ms for males and below 460 ms for females. This can be prolonged for many reasons (drugs, congenital disorders, electrolyte imbalances, etc.) and has been used to indicate a variety of pathologies (Viskin 2009). Prolonged QT intervals indicate delayed cardiac repolarization and increased vulnerability to life-threatening malignant ventricular arrhythmias.

Genetics of the QT Interval

More than 17 genes have been linked to prolonged QT. Two well-described congenital syndromes include Romano-Ward syndrome (autosomal dominant) and Jervell and Lange-Nielsen syndrome (autosomal recessive form with deafness). Short QT syndrome is very rare and is described as a QT interval below 330 ms (Postema and Wilde 2014). The most clinically significant concern in a patient with prolonged QT is development of fatal arrhythmias such as torsade de pointes and subsequent ventricular fibrillation. Lehtinen et al. looked at common single nucleotide polymorphisms (SNPs) in sodium and potassium channel genes in those with diabetes and found that genetic variants may be associated with the QT interval duration (Lehtinen et al. 2009). The relationship between genetic variants and QT interval was reproduced in a Finnish study surveying 15 common SNPs (Noseworthy et al. 2011).

Early ECG Changes in Diabetes

Even before cardiac involvement is clinically evident in diabetes, fibrotic changes in the basal area of the left ventricle have been observed. Inverted T waves in lead I and aVL indicate fibrosis of the midventricular area, for example. Sinus tachycardia, ST segment changes, and QT dispersion have also been observed early in the course of diabetes (Stern and Sclarowsky 2009). The EURODIAB Insulin-Dependent Diabetes Mellitus Complications Study (EURODIAB IDDM) followed 3250 subjects with type 1 diabetes for an average of 30 years and found that left ventricular hypertrophy was as much as three times more prevalent in those with diabetes than the general population (Giunti et al. 2005) (Fig. 2). They also found that the QT interval duration was independently associated with HbA1c (Veglio et al. 1999).

Glycemic Variability and the QT Interval

Glycemic variability and chronic sustained hyperglycemia are significant risk factors for diabetic complications and are linked to increased mortality (Singleton et al. 2020e). Prolonged QTc intervals indicate a prolonged cardiac repolarization which

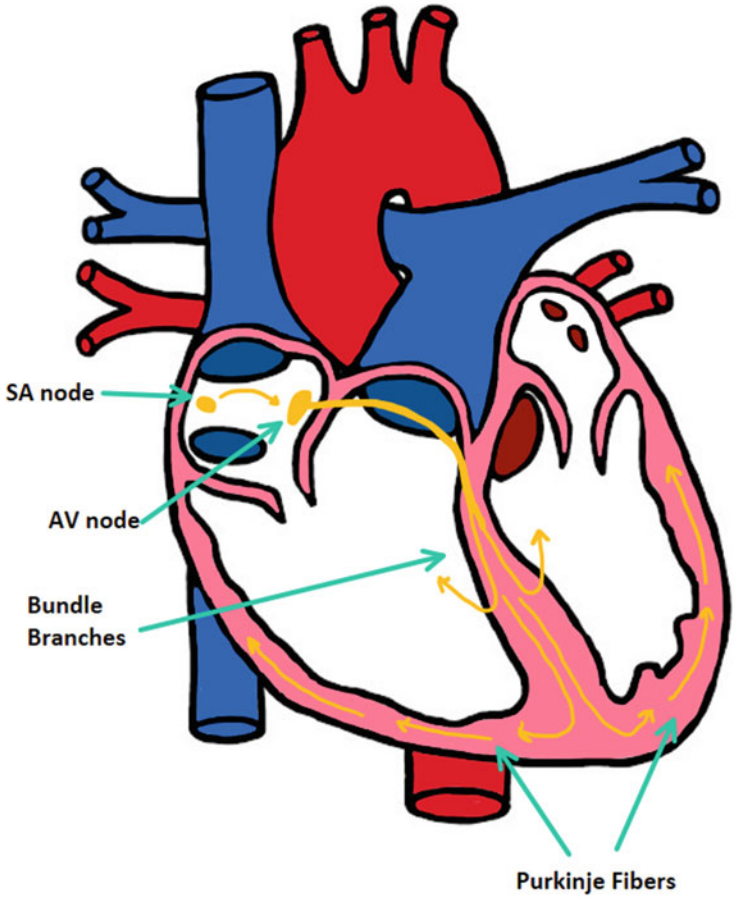

Fig. 2 Cardiac conduction system. This figure illustrates electrical circuit components of the conduction system of the heart. Pacemaker cells in the SA node fire first and the signal travels through the AV node (bundle of His), bundle branches, and Purkinje fibers

can cause cardiac arrhythmias and sudden cardiac death, which are highly prevalent in type 2 diabetes (Yang et al. 2017). In a study of 2904 individuals with type 2 diabetes, those with prolonged QTc interval had greater post-prandial glucose and fasting plasma glucose levels (Su et al. 2017). A separate study of 18 subjects found that there were several alterations in ECGs in a hypoglycemic state (Laitinen et al. 2008). Hypoglycemia is thought to trigger sympathetic overactivation and catecholamine secretion which causes electrolyte disturbances, dysregulation of atrioventricular conduction, ventricular depolarization, and ventricular repolarization. Catecholamines such as epinephrine have been shown to directly prolong the QTc interval and induce T wave flattening (Struthers et al. 1983). A relative shortening of the PR interval, depression of the ST segment, and decreased T wave area were also observed in the setting of hypoglycemia.

QT Interval in Diabetes Mellitus

Various studies have attempted to categorize markers of cardiac disease as predictors of mortality. The Strong Heart Study showed that after correction for age, sex, and other risk factors, ST segment depression and QTc interval predicted all-cause mortality in American Indians (Okin et al. 2000, 2004). The Diabetes Heart Study showed that the QTc interval is an independent predictor of all-cause mortality in those with type 2 diabetes (Cox et al. 2014). This study also showed that the QT duration correlates with the amount of coronary artery calcium and is driven by QRS rather than the JT interval and that QRS duration is associated with all-cause mortality in type 2 diabetes (Nelson et al. 2008; Singleton et al. 2020a). As diabetes progresses, microvascular complications such as nephropathy and retinopathy arise. A study of 219 patients in Tokyo with type 2 diabetes found that as the severity of microvascular complications increased, the QTc prolongation also increased (Kobayashi et al. 2018).

QT Interval and Sudden Cardiac Death

Prolongation of the QT interval has been associated with increased risk for sudden cardiac death in several population-based cohort studies (Noseworthy et al. 2011). The association between QT abnormalities and acute and chronic cardiac ischemia has been well defined (Peters et al. 1990; Dekker et al. 2004). A study by O'Neal et al. examined the risk of SCD for each QT interval component (R-wave onset to R-peak, R-peak to R-wave end, ST-segment, T-wave onset to T-peak, and T-peak to T-wave end). In this study, they found that prolongation of the T-wave onset to T-peak component had the strongest association with SCD (O'Neal et al. 2017). The Oregon Sudden Unexpected Death study also found a significant correlation between QTc prolongation and sudden cardiac death in those with diabetes (Chugh et al. 2009). It has also been shown that analysis of the QT interval is superior to the RR interval and ankle brachial pressure index in predicting cardiac death in diabetes (Rana et al. 2005).

QT Interval and Atrial Fibrillation

There is also an emerging body of evidence that the QT interval may be a marker of heightened risk of atrial fibrillation, especially in patients with diabetes (Mandyam et al. 2013). Atrial fibrillation is an irregular heartbeat in which the atrium and ventricles do not contract in a coordinated manner. This is particularly important, as atrial fibrillation is independently associated with increased mortality (Ehrhardt-Humbert et al. 2020; Singleton et al. 2020d) and patients with diabetes already have a heightened risk of atrial fibrillation, and there are risk factors for incident atrial fibrillation that are modifiable (Mayl et al. 2020; Singleton et al. 2020c, 2021a),

as well as some that are not (Dhaliwal et al. 2020). Atrial fibrillation increases the risk of developing blood clots which can result in stroke, heart attack, and SCD.

QT Dispersion

QT dispersion is defined as the difference between the maximum and minimum QT interval. QT dispersion has been proposed as a marker for malignant ventricular arrhythmias and is more prevalent in long QT syndromes (Yamaguchi et al. 2003). Sawicki et al. found in long-term follow-up that QT dispersion is an independent predictor of cardiac, cerebrovascular, and total mortality in non-insulin-dependent diabetes (Sawicki et al. 1998). A cross-sectional study of 501 patients with type 2 diabetes defined the prevalence of prolonged QT interval and QT dispersion as well as their clinical and metabolic predictors. It was found that the prevalence of prolonged QT interval and QT dispersion are 44.1% and 3.6%, respectively. There was a significant relationship between the QTc duration and QTc dispersion ($r = 0.36$). It was also found that those with prolonged QT interval had a higher age, BMI, prevalence of coronary heart disease, retinopathy, and polyneuropathy (Ninkovic et al. 2016).

Pathologic Q Waves: Diabetic Risk of Silent Myocardial Infarction

Pathologic Q waves are a negative deflection of the QRS complex toward the negative pole of the lead axis and are observed in myocardial injury, ventricular enlargement, altered ventricular conduction, and physiologic and positional effects. Clinicians utilize pathologic Q waves for detection of myocardial damage such as a myocardial infarction, which is one of the leading causes of death in diabetes. A pathologic Q-wave on ECG can be indicative of prior ischemic damage to the myocardium and may suggest a higher-risk patient population (Singleton et al. 2020b). Diabetes is complicated by development of neuropathy and more severe atherosclerosis which creates the perfect setup for an asymptomatic ischemic cardiac event, or a silent myocardial infarction (SMI). Those with diabetes have impaired nociception and are less likely to present with chest pain when having a heart attack. One study found that SMI with significant lesions occurs in over 20% of male type 2 diabetic patients who are asymptomatic (Janand-Delenne et al. 1999). Identifying SMI in diabetes is important as those individuals may benefit from therapies to mitigate risk.

REGARDS Study

Several studies have further strengthened the link between diabetes and poor cardiovascular health. The Reasons for Geographic and Racial Differences in Stroke (REGARDS) study is a longitudinal study of 30,000 African-American and

Caucasian adults to determine causes for excess stroke mortality (Howard et al. 2005). A secondary analysis of the data provided risk ratios between elevated fasting glucose levels and poor cardiovascular health. Ideal cardiovascular health was associated with a dose-dependent lower risk of diabetes with normal fasting glucose but not for impaired fasting glucose levels (Joseph et al. 2019).

Applications to Prognosis and Other Diseases or Conditions

The QT interval is obtained through noninvasive means and can be obtained in both outpatient and emergent settings. While reading an ECG often requires a cardiologist, a computer readily interprets the duration of the QT interval. Any physician is able to delineate a prolonged QT interval on such studies, making this biomarker applicable and available to most clinical settings. In the same way that other calculators and biomarkers help to stratify risk in other diseases, we believe the QT interval can play an integral part in stratifying risk of all-cause mortality in those with diabetes. Additionally, because prolonged QT interval and increased QT dispersion could signify subclinical heart disease, one could utilize the QT interval as a means of assessing cardiac damage in those with increased risk of heart disease. For example, in persons with history of smoking, hyperlipidemia, and hypertension, regular surveillance of the QT interval may stratify patients to a higher risk category of mortality due to heart disease than previous indicators. In those patients, clinicians can utilize shared decision-making regarding the use of medications or other interventions to further reduce the risk of heart disease and all-cause mortality.

Mini-Dictionary of Terms

- **Atrial Fibrillation:** Irregular heartbeat in which the atrial chambers beat out of coordination with the ventricles.
- **Diabetic Neuropathy:** Hyperglycemia damages the blood vessels that supply the nerves. Long nerves such as those supplying the feet are often damaged first.
- **Diabetic Retinopathy**: Hyperglycemia-induced damage to microvasculature supplying the retina, most often manifested as increased proliferation of vasculature of the eye. This leads to blurry vision, vision loss, and eventually blindness.
- **QT Interval:** Segment of the QRS-T wave corresponding to the duration of repolarization of the ventricles from the apex to the base of the heart.
- **QT Dispersion:** Difference between minimum and maximum QT interval duration.

Key Facts of QT Interval

- The QT interval corresponds to depolarization (ventricular systole, isovolumetric contraction) and repolarization of the ventricles (isovolumetric relaxation).
- Typical QT interval durations are below 440 ms for men and 460 ms for women.

- Several genes are correlated with prolonged QT intervals.
- Prolonged QT intervals translate to delayed repolarization of the heart which increases the risk of fatal arrhythmias such as torsades de pointes.
- Congenital short QT syndrome is rare and associated with paroxysmal atrial fibrillation, ventricular fibrillation, and sudden cardiac death.

Key Facts of Atrial Fibrillation

- Irregular heartbeat occurs when the atria and ventricles are not electromechanically coupled.
- Decreased coordinated contraction of the atrium leads to decreased laminar flow exiting the atrium. Stagnant blood can form clots which can travel to the brain, extremities, or other parts of the body.
- Due to risk of clots, the CHA_2DS_2-VASc Score is used to calculate the risk of stroke in those with afib. Those at high risk are placed on oral anticoagulation medication such as warfarin.
- Stretching of the atrium due to increased blood return from the lungs or decreased blood flow exiting the left side of the heart can predispose the atrium to atrial fibrillation.
- Atrial fibrillation can occur in brief episodes, known as paroxysmal atrial fibrillation, or can be a chronic condition.
- When indicated, anti-arrhythmogenics such as amiodarone and procainamide are used to slow electrical conduction through the heart.
- Rate control medications such as calcium channel blockers and beta blockers are used to slow the heart rate.

Key Facts of Malignant Arrhythmias

- Malignant arrhythmias are those that can result in sudden cardiac death.
- These include ventricular fibrillation, ventricular tachycardia, and torsades de pointes.
- These occur from myocardial disease, structural cardiac abnormalities, congenital arrhythmias, electrolyte imbalances, drugs, and some infections.
- Patients experiencing one of these malignant arrhythmias are hemodynamically unstable and quickly develop cardiovascular collapse.
- Treatment of these may include urgent defibrillation, CPR, and electrolyte correction.

Key Facts of Sudden Cardiac Death

- Sudden cardiac death (SCD) is not a myocardial infarction (blockage of coronary arteries causing oxygen deprivation to the heart).

- SCD occurs when electrical activity of the heart becomes fast and irregular, such as in a malignant arrhythmia.
- The heart experiences uncoordinated contraction between the atrium and ventricles. The chambers flutter or quiver rather than contract, leading to decreased blood flow.
- The heart rate becomes too fast to allow for sufficient filling of the heart between contractions, resulting in reduced cardiac output.
- A person experiencing this may have symptoms of dizziness, light-headedness, and increased rate of breathing and may lose consciousness.
- Emergent CPR and defibrillation are needed for resuscitation.

Summary Points

- Diabetes mellitus is associated with increased risk of cardiovascular disease and all-cause mortality.
- Glycemic variability causes stress on cardiac myocytes, leading to structural damage and conduction disturbances.
- Diabetes is associated with increased prevalence of prolonged QT interval and increased QT dispersion.
- Prolonged QT interval predisposes the heart to malignant arrhythmias including ventricular tachycardia, ventricular fibrillation, and torsades de pointes.
- Malignant arrhythmias can result in sudden cardiac death.
- Prolonged QT interval is associated with increased risk of atrial fibrillation which leads to increased risk of stroke and heart attack.
- Regular surveillance of QT interval in those with diabetes may aid in stratifying risk of all-cause mortality in those populations.

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Biomarkers of Myocardial Fibrosis in Diabetes, Echocardiography, and Magnetic Resonance Imaging

40

Per Lav Madsen

Contents

Introduction 823
Heart Disease in Diabetes 823
Myocardial Fibrosis in Diabetes 827
Cardiac Metabolism in Diabetes 827
The Heart's Interaction with the Peripheral Circulation 827
The Myocardial Extracellular Volume 828
Animal Models of Cardiac Fibrosis in Diabetes 828
Magnetic Resonance Imaging of Fibrosis and the Extracellular Volume 829
The Two CMR Techniques of Fibrosis Quantitation 829
Tissue T1 Times 830
Gadolinium Contrast 831
Myocardial Blood Flow Determination with CMR 832
Determination of the Extracellular Volume 832
The Late Gadolinium Enhancement Technique 832
CMR Determination of Fibrosis in Cardiomyopathies 834
Ischemic Versus Non-ischemic Cardiomyopathy with LGE 834
Extracellular Volume and Fibrosis in Diabetes by CMR 835
Cardiac Fibrosis and Extracellular Volume in Patients with T2DM 835
Relation of Cardiac Fibrosis to Markers of Diastolic Function 836
Myocardial Blood Flow in T2DM 837
The Relation of Fibrosis to Myocardial Blood Flow 837
Looking to the Future 840
Longitudinal Studies 840
CMR for Screening of DM Patients? 840
Validation of Serum Biomarkers of Fibrosis 841
Applications to Prognosis 841

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Key Facts of Diabetic Cardiomyopathy 842
Key Facts of Cardiac Magnetic Resonance Imaging of Fibrosis 843
Mini-Dictionary of Terms 843
Summary Points 844
References 845

Abstract

Diabetes mellitus (DM) is associated with cardiomyopathy. Cardiomyopathy in DM can be from ischemic heart disease or related purely to DM-related myocardial changes. The non-ischemic variant of cardiomyopathy ("diabetic cardiomyopathy") is in animals related to myocardial hypertrophy, vascular rarefaction, and expansion of the extracellular volume by glycated hemoglobin and fibrosis, and recent echocardiography and magnetic resonance imaging studies in humans are in line with this. In patients with type 2 DM, the left ventricle becomes stiff with concentric remodeling. Myocardial blood flow can increase only threefold during stress, whereas it is normally fivefold. Normally 25% of the myocardium is taken up by the extracellular volume, but in T2DM patients this fraction is increased, in some patients to values >40%. Myocardial fibrosis and maximal myocardial blood flow are inversely correlated, and in some patients virtual "islands of fibrosis" can be detected. These areas probably reflect the fibrotic islands initially described by Rubler et al. (Am J Cardiol 30:595–602, 1972) in the autopsy studies defining "diabetic cardiomyopathy." Longitudinal studies are now pursued to determine the importance of myocardial hypoperfusion and myocardial fibrosis.

Keywords

Diastolic function · Echocardiography · Heart failure · Heart failure with reduced ejection fraction · Heart failure with preserved ejection fraction · Magnetic resonance imaging · Myocardial fibrosis · Myocardial extracellular volume · Myocardial blood flow · Gadolinium contrast · Type 1 diabetes mellitus · Type 2 diabetes mellitus

Abbreviations

AGE	Advanced glycation end product
CMR	Cardiovascular magnetic resonance imaging
DM	Diabetes mellitus
ECV	Extracellular volume
GLP1	Glucagon-like peptide-1
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
IHD	Ischemic heart disease
LGE	Late gadolinium hyperenhancement
LV	Left ventricle

LVEF	Left ventricle ejection fraction
MOLLI	Modified Look-Locker inversion recovery pulse sequence
MRI	Magnetic resonance imaging
SGLT2	Sodium-glucose transport protein 2
ShMOLLI	Shortened MOLLI
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

Introduction

Heart Disease in Diabetes

Diabetes mellitus (DM) is associated with among others kidney failure, retinopathy, and peripheral neuropathy, but the significantly shortened life span of a person with DM is largely associated with the induced heart disease. The heart disease in DM can be divided into two forms: ischemic heart disease (IHD) and a non-ischemic heart disease not dependent on DM-related acceleration of the coronary atheromatosis process. A DM patient with heart disease often suffers from both, but can suffer from one independently of the other. DM has long been known to be associated with IHD, and guidelines describe DM as a cardiovascular disease necessitating treatment with antihypertensives, statins, and a vigilant focus on development of significant myocardial ischemia. Even if IHD does not develop, however, DM patients may develop heart failure (HF) from myocardial changes brought about by DM itself. The three overarching phenotypical changes that can be seen in animal and autopsy studies are myocardial vascular rarefaction, left ventricle (LV) hypertrophy, and disperse myocardial fibrosis. These facts are far less recognized in clinical medicine than the well-known HF in DM-associated IHD. DM is often associated with hypertension that in itself is associated with both myocardial hypertrophy and diastolic dysfunction (and further acceleration of the atheromatosis process). While DM may indeed in older age, and notably so if associated with IHD, be associated with HF with reduced ejection fraction (HFrEF; left ventricle (LV) ejection fraction <40%), most patients with DM do not readily develop overt pump failure with dilated poorly contracting ventricles, but later develop a HF that despite a normal or near-normal ejection fraction leaves patients with signs of heart failure notably from diastolic (filling) problems of their stiff and thickened myocardium.

Heart Failure with Preserved Ejection Fraction

This latter situation is overarchingly referred to as HF with preserved ejection fraction (HFpEF). The sodium-glucose transport protein 2 (SGLT2) antagonist empagliflozin (well-known to be of importance in T2DM) has recently been shown to be of benefit for patients with HFpEF both with and without DM (Anker et al. 2021). In general, however, the overarching term of HFpEF for all patients with signs of HF but seemingly normal pumping chamber function is not always helpful as has before the SGLT2 antagonist trial been demonstrated indirectly in a number of

previously futile randomized studies. In retrospect, such studies have probably lumped patients with very different kinds of LV filling problems. Thus, a number of patients classified under this overarching term of HFpEF probably have very different reasons for their respective HF symptoms and signs. There is a large difference between a patient with signs of diastolic dysfunction based on amyloidosis of the heart and a patient with diastolic dysfunction based on myocardial disperse fibrosis and hypoperfusion from small vessel disease. And the treatment of the underlying cause of their common symptoms should differ. While echocardiographic biomarkers of HFpEF have long been demonstrated (Jørgensen et al. 2019), overall treatments of HFpEF have been disappointing, and it now seems timely to more precisely determine underlying reasons for HFpEF in different patient populations. In comparison with HFrEF patients, patients with HFpEF do not develop "hard endpoints" (e.g., myocardial infarction, admittance to hospital with congestion, or death) as early and frequently. Hence, there is a grave need to develop and evaluate biomarkers of myocardial hypoperfusion and notably the underlying fibrosis in order for immediate evaluation of DM patients suspected of HFpEF and for interventional randomized studies to be able to address these underlying phenotypic aspects of heart disease in patients with DM. Although shorter-term success may be obtained with treatment related to final pathophysiological pathways, medium- and longer-term treatment success must be expected to differ significantly and may successfully require careful considerations of early underlying myocardial pathophysiological causes.

Imaging Biomarkers of Myocardial Function in Diabetes and HFpEF

In this chapter, imaging biomarkers of myocardial hypoperfusion and fibrosis with cardiovascular magnetic resonance imaging (CMR) are reviewed, and their relation to noninvasive biomarkers of cardiac filling problems is evaluated. The studies are mainly concerned with recent CMR studies that have opened up a door for concomitant evaluation and quantification of two important overarching pathophysiological factors associated with the stiffened and hypertrophic heart noted in patients with DM: myocardial hypoperfusion and myocardial disperse fibrosis expanding the extracellular volume. With such noninvasive imaging biomarkers, it will be possible to detect the underlying causes of suspected HF in a patient with DM, and in the future hopefully make it possible in randomized studies to evaluate the impact of chosen interventions on myocardial blood flow and fibrosis, and implicitly eventually initiate treatment more relevant for the particular patient in question. Initially the underlying causes of diabetic heart disease as demonstrated in animal studies and previous human studies are shortly reviewed and explained. Thereafter the CMR technique by which myocardial fibrosis is evaluated is reviewed and lastly recent CMR studies on myocardial perfusion and fibrosis in T2DM patients are presented. Reviewing the past, but looking to the future, further studies including longitudinal studies and associated studies of serum biomarkers of fibrosis are discussed.

Epidemiology of Diabetic Heart Disease

HF is strongly associated with DM. The Framingham Heart Study early demonstrated 2.4- and 5.1-times increase in the incidence of HF in men and women with DM respectively in comparison with an age-matched cohort adjusted for hypertension, obesity, ischemic heart disease, and dyslipidemia (Kannel and McGee 1979; Kannel 2011). In general, in HF cohorts, including both HFrEF and HFpEF, the prevalence of DM ranges from 10% to 47% (From et al. 2006; Shindler et al. 1996; Cleland et al. 2003; Dei Cas et al. 2015; Sandesara et al. 2018). The prevalence of DM is higher in patients hospitalized with HF, with some reports of >40% (Greenberg et al. 2007). In patients with DM, the prevalence of HF is between 9% and 22%, which is four times higher than in the general population, and the prevalence is even higher in patients with DM who are older than 60 years (Nichols et al. 2004; Bertoni et al. 2004; Boonman-de Winter et al. 2012; Thrainsdottir et al. 2005). Thus, it has long been known that DM is a significant risk factor for cardiovascular disease, but the specific cardiovascular changes seen with DM are still incompletely understood and hence difficult to treat. With glucagon-like peptide-1(GLP1) receptor agonists and SGLT2 receptor antagonists, there is light at the end of the tunnel, but so far conventional antiglycemic therapy has added little improvement if any to cardiovascular disease of patients with DM, and even after treatment with antihypertensives and statins patients with DM still have a significantly increased risk of myocardial infarction and HF.

Diabetic Cardiomyopathy

DM, on its own, is associated with a cardiomyopathy, often simply termed "diabetic cardiomyopathy," not founded on the acceleration of IHD. The association of heart disease with DM had been noted before, but as the first, the Danish physician Lundbæk in 1954 specifically noted vascular dysfunction in patients with DM (Lundbaek 1954), and in 1969 he as the first suggested a "diabetic cardiomyopathy" (Lundbaek 1969), i.e., a variant of heart disease related more to DM per se than the often accompanying atherosclerotic and IHD. In 1972, Rubler et al. on autopsy of DM patients proved Lundbæk right (Rubler et al. 1972). Rubler et al. autopsied patients with DM, who had died from congestive HF with edema of the lungs and dilated hearts, and demonstrated that even though these patients had significant myocardial "islands" of fibrosis, they did not have significant atheromatosis of the coronary arteries. Instead Rubler et al. found that these patients too suffered from myocardial hypertrophy and found that the "islands" of myocardial fibrosis were associated with microvascular wall thickening from accumulation of acid mucopolysaccharides ("advanced glycation end products," AGEs). From then on it was understood that HF in DM could in fact be associated with myocardial fibrosis, not related to significant ischemia from an otherwise often accompanying IHD or indeed previous myocardial infarctions.

Treatment of Diabetes in Relation to Cardiomyopathy

It sometimes seems as if these early studies have been forgotten, and with previous techniques fibrosis of the heart could only be demonstrated in autopsy and animal studies. Hence, early clinical and imaging studies of cardiovascular disease in DM have often focused on the accompanying IHD. This has not been irrational since DM is indeed associated with a significant overrepresentation of morbidity and premature mortality from IHD, and IHD is indeed a prevalent risk in DM. Further studies using PET and myocardial scintigraphy have demonstrated often widespread myocardial perfusion defects even in DM patients not presenting with angina. DM is still associated with significant coronary disease, but it seems that adhering to modern era guidelines, the risk of IHD may be declining in DM, and treatment of such with notably antihypertensive medication and statins has significantly prolonged lives in patients with DM. The STENO2 studies have demonstrated that overall the more successful treatment of T2DM patients is not glucose-lowering therapy by insulin but a multifactorial intervention notably involving statins and antihypertensives (Gaede et al. 2003). Improved treatment of coronary atheromatosis in patients with DM is indeed warranted, but even after treatment of such, patients with DM still end up with HF. Such HF may, as shown by Rubler et al., be classic HF with dilated weakly contracting ventricles (as suggested from the report today, Rubler's patients would probably have been diagnosed with HFrEF), and DM is indeed related to "non-ischemic" HFrEF (i.e., HFrEF with normal coronary arteries) (Bertoni et al. 2004; Kannel 2011), but this is usually only seen with old age. Reports of HF in DM mainly describe a cardiac phenotype with a small left ventricle (LV) with concentric hypertrophy, a normal or near-normal LVEF, and elevated LV filling pressures (diastolic dysfunction) (Jørgensen et al. 2019). With signs of HF, these pts. are labeled as having HFpEF (For a comprehensive review, please see Ng et al. 2021). Compared with DM-associated myocardial dysfunction, the "obese phenotype" of HFpEF is less well recognized and little explored, but several studies have demonstrated an association between obesity and LV structural and functional changes (Ng et al. 2021), and part of the problem may relate to not only DM but also to obesity changing notably the afterload faced by the ventricles of the heart by increased arterial blood pressure and lowered distensibility of conductance arteries. Obesity is an important risk factor for T2DM, and in the light of newly established treatments of obesity (notably gastric bypass and the GLP1 receptor analogues), it is important to understand to what degree cardiovascular changes seen with T2DM are associated with DM per se or the added cardiovascular burden of obesity is associated with for example stiff peripheral conductance arteries and hence the added afterload for the LV.

Treatment of HFpEF

The distinction between HFrEF and HFpEF is important since treatment of HFrEF has for long been well-established with repeated and still ongoing successes, but randomized controlled trials in HFpEF have hitherto been disappointing, with positive influence only from perhaps treatment with spironolactone and now firmly established from the SGLT2 antagonist empagliflozin (Anker et al. 2021). GLP1 receptor agonists and SGLT2 antagonists are important new treatment options in obesity and T2DM, but

for most patients with diabesity-related stiffening of the LV, no specific treatment is known, except for advice on exercise, weight loss and by halting development of IHD with statins, smoking cessation, and antihypertensive medication. To address diabesity-related heart disease, it will be important to understand the underlying phenotypic causes in more detail and preferably so in larger populations where follow-up studies with adequate statistical power are possible.

Myocardial Fibrosis in Diabetes

Cardiac Metabolism in Diabetes

Studies of underlying factors have focused on IHD that in itself can induce diastolic dysfunction and the specific consequences of hyperglycemia, insulin resistance, increased free fatty acids, tissue inflammation, oxidative stress, and activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system (for a comprehensive review, please see Jia et al. 2016). In short, hyperinsulinemia and oxidative stress promote expression of "hypertrophy genes" and reduce nitric oxide bioavailability and sarcoplasmic reticulum Ca^{2+} pump activity. The latter give rise to a stiff isoform of the cytoskeletal "scaffolding" protein titin, and increased interstitial collagen cross-linked by AGEs. Insulin resistance makes adenosine triphosphate production more reliant on fatty acid metabolism that requires a higher oxygen uptake than glucose metabolism does, and the myocardial adenosine triphosphate to phosphocreatine ratio is lowered in T2DM and obese patients (Scheuermann-Freestone et al. 2003). This places diabesity patients with hypertrophy and myocardial vascular rarefaction at jeopardy. In patients with autonomic neuropathy, myocardial contractile and relaxation kinetics are reduced, and some have impaired heart rate increment with exercise. Also, impaired cardiac interaction with the peripheral circulation ("ventriculo-arterial coupling") poses an additional risk (Ng et al. 2021; Kyhl et al. 2021).

The Heart's Interaction with the Peripheral Circulation

Thus, in diabesity cardiac function must be related to the heart's interaction with aspects of the peripheral circulation notably the blood pressure (often high) and conductance artery distensibility (often low) both independently affecting afterload for the left ventricle, and the latter negatively influencing left ventricular filling even in normal subjects without arterial hypertension or myocardial hypertrophy (Kyhl et al. 2021). Cardiac output and blood volume are suggested to be large, but on the other hand the blood volume of the heart and lungs may be compressed from excessive pressure from the outside (high pulmonary pressure, stiff thorax, high epi- and pericardial fat content) (Obokata et al. 2017). It is little studied how the cardiac output is redistributed to peripheral organs, and the general fitness level is often not adequately taken into consideration. The problem is that T2DM patients have stiff hearts, but it cannot be determined why.

The Myocardial Extracellular Volume

Typically, myocardial tissue is composed of approximately 75% myocardial intracellular tissue, which consists of myocardial cells and blood cells, whereas 25% consists of the extracellular volume (ECV), i.e. the space between all cells, including plasma in the vasculature. The myocardial interstitial space accounts for approximately 90% of the ECV and is composed of fibrillary proteins (elastin and collagen I and III), non-fibrillary proteins (aminoglycans, fibronectin, and laminin), bioactive proteins (transforming growth factor beta and matrix metalloproteinases), and cells (fibroblasts and resident monocytes). Animal studies suggest that disperse fibrosis of the myocardial ECV is in fact an important part of "diabetic cardiomyopathy." Patients with DM, but without significant macrovascular disease, are known to display microvascular small intramyocardial arteries and arterioles. In DM, collagen is deposited perivascularly with thickening and sclerosis of smaller myocardial arteries with little possibility for dilatation.

Numerous histopathologic studies have now demonstrated that cardiac fibrosis in DM patients can occur independently of coronary atherosclerosis or hypertension. Regan and co-workers (1977) showed that patients with adult-onset DM do indeed exhibit extensive perivascular, interstitial, and even replacement fibrosis, even in the absence of hypertension or coronary artery disease. Myocardial fibrosis in patients with DM is often accompanied by cardiomyocyte hypertrophy and by evidence of microvascular abnormalities, including thickening of the capillary basement membrane (Kawaguchi et al. 1997). DM-associated interstitial fibrosis has been described in both T1DM and T2DM, is associated with accumulation of both type I and III collagen, and involves both the left and right ventricles (Nunoda et al. 1985). Relations between DM-associated fibrosis and cardiac function have not been systematically investigated, but in a study examining biopsies from patients with HF in the absence of coronary disease, DM was associated with increased collagen levels only in patients with reduced ejection fraction (van Heerebeek et al. 2008). DM is also associated with accentuation of fibrotic changes in patients with other cardiac conditions. Thus, in patients with aortic stenosis, diabetes is associated with worse myocardial stiffness and increased myocardial collagen content (Falcao-Pires et al. 2011).

Animal Models of Cardiac Fibrosis in Diabetes

Animal models of T1DM provide strong support to the association between T1DM and myocardial fibrosis, and in both mice and rats, streptozotocin-induced T1DM is associated with interstitial myocardial fibrosis, accompanied by cardiomyocyte hypertrophy, induction of pro-fibrotic and hypertrophy-associated genes, and microvascular rarefaction (Cavalera et al. 2014; Shen and Bornfeldt 2007; Ares-Carrasco et al. 2009). Pro-fibrotic effects of T1DM on the myocardium have also been demonstrated in larger animal models including mongrel dogs (Regan et al. 1981) and rhesus monkeys (Haider et al. 1981). Cardiac fibrosis has also been documented

in experimental animal models of T2DM. Db/db mice express a truncated leptin receptor and are resistant to the central effects of leptin and develop severe obesity and T2DM at 1–2 months of age. In db/db mice, cardiac fibrosis accompanied by cardiomyocyte hypertrophy and diastolic dysfunction is seen at 4–6 months of age (Biernacka et al. 2015; Huynh et al. 2012). Genetic models of T2DM in the rat also exhibit cardiac fibrosis. Zucker rats have a leptin receptor missense mutation and develop severe obesity and insulin resistance. These animals develop perivascular myocardial fibrosis, associated with cardiomyocyte hypertrophy and diastolic dysfunction (Fredersdorf et al. 2004).

Magnetic Resonance Imaging of Fibrosis and the Extracellular Volume

The role of diffuse reactive fibrosis and overt myocardial scarring characterized by accumulation of myocardial collagen in the extracellular spatium has long been identified as an important factor in the etiologies of different cardiomyopathies with either reduced or preserved ejection fraction. The myocardial interstitium has therefore become a subject of intense focus in most cardiomyopathies including diabetic cardiomyopathy, and techniques have been vigorously sought to visualize and quantitate such fibrosis and the associated expansion of the extracellular spatium. The diffuse nature of the structural changes associated with diffuse fibrosis made it difficult to detect except in pathology studies and in invasive studies using biopsies. Except in special circumstances, however, biopsies have never really been a viable option in everyday clinical patient management, and detection and quantification of fibrosis from biopsies are prone to sampling error since fibrotic areas do not necessarily affect all of the myocardium. With CMR, especially so with the use of gadolinium for contrast, it has become possible to noninvasively image and quantify the myocardial interstitium and hence to detect fibrosis. It is not an overstatement to say that the technique(s) of CMR determination of fibrosis has revolutionized imaging cardiology and the interest in applying CMR with gadolinium contrast for clinical and research studies in cardiology has long increased (near-) exponentially.

The Two CMR Techniques of Fibrosis Quantitation

Determination of cardiac fibrosis with CMR rests largely on two related techniques. Either virtual "islands of fibrosis" (scars) of the myocardium are visualized and quantified with the so-called late gadolinium hyperenhancement (LGE) technique or diffuse fibrosis (increased ECV) is quantified with so-called "T1 mapping" technique. With the LGE technique, "islands of fibrosis" are depicted (usually on "white-on-black" images), and with the T1 mapping techniques, disperse myocardial fibrosis is quantitated pixel by pixel (square in image) and visualized on color-coded maps. The techniques are related but are for pedagogical reasons best explained as two different techniques. Both techniques build on the principle that

different tissues (e.g., normal vs. fibrotic or scarred myocardium) have different T1 times. The following simple though still slightly tedious explanation of the differences in MR acquisition techniques can be omitted, as long as it is remembered that these two overarching principles exist and are now both used extensively in clinical studies and in cardiac research on cardiac fibrosis. Different well-described cardiomyopathies display areas of LGE in different patterns. These patterns are often quite easily discernible laying the foundation for phenotypic classification of the cardiomyopathy of a patient presenting with HF. For example, a patient with ischemic cardiomyopathy will show subendocardial LGE, whereas a patient with myocarditis will show subepicardial LGE areas. The T1 mapping technique often complements the LGE technique and is increasingly used in the study of cardiomyopathies where only diffuse fibrosis is present but no discernible phenotypic pattern using the LGE technique may be present.

Tissue T1 Times

In general, the techniques rely on the fact that different tissues either naturally or after administration of a contrast will have different T1 times. Protons that are "flipped" by a radio-signal in the longitudinal magnetic field of an MRI scanner will return after the radio signal has ceased to their normal position with a tissue-specific T1 time. The duration of the T1 time will depend on the "ease" by which the protons are allowed to flip back, as the relative ease is related to how easily they are allowed to give up their heightened energy level to the surroundings. The recalibration of proton spins after the radio-frequency pulse has ceased quite closely follows an exponential function, and hence can be characterized by a time constant. The T1 time is the time after which the system has recalibrated approximately 63%. The T1 time length measures what is referred to as the longitudinal or "spin-lattice" (since it is mainly related to the protons interacting with the surroundings, in general referred to as the lattice) relaxation time. If the T1 time of a tissue can be determined (or if the difference between T1 times of different myocardial tissue samples is used to produce contrast in an image), it will bring about important information of the tissue composition and hopefully a quite precise phenotyping of the myocardium.

Gold-standard determination of T1 time is in theory interesting, but such a gold-standard determination is time-consuming, since it requires a large number of so-called inversion pulses. In general such a study may take more than 20 min. In 1970, however, Look and Locker proposed a method to measure T1 relaxation times quite closely to real T1 times by acquiring data successively after magnetization inversion with a relatively simple scheme. The scheme proposed by Look and Locker (1970) was much less time-consuming than the gold-standard technique and therefore suddenly made T1 determination of clinical relevance. The method originally suggested by Look and Locker has since been refined, and acquisition times have been shortened further. The latter is important for research and everyday clinical work since acquisitions will have to be performed during breath-holds (otherwise, diaphragmatic movements will make the heart move too much) and

some patients with heart or pulmonary disease struggle with long breath-holds. Today a number of different pulse sequences exist that determine T1 time with short acquisition times. One of the most popular is the modified Look-Locker inversion recovery pulse sequence that allows measurement of T1 times in a single breath-hold over 17 successive heartbeats (Higgins and Moon 2014). This may still be too long for some elderly breathless patients to endure (i.e., this sequence will take 17 s at a heart rate of 60 bpm), and often variations are used with shortened breath-hold durations and reduced sensitivity to heart rate, such as the "5(3)3 scheme" (describing how many and how often during a heartbeat pulses are applied). The shortened MOLLI (ShMOLLI) scheme uses sequential inversion recovery measurements with a single breath-hold of only nine successive heartbeats and a conditional fitting algorithm to account for the short recovery period between inversion pulses. Such shortened schemes make T1 mapping useful in most patients from outpatient clinics.

Gadolinium Contrast

With T1 mapping phenotypically different areas of the heart are discerned by mapping of each voxel's (each cube of the myocardium) T1 time. T1 mapping is performed both without contrast ("native T1 mapping") and after administration of gadolinium contrast. It is important to maximize the difference between two adjacent (but phenotypically truly different) tissues and hence lower interrater discrepancy (noise). Significantly larger variations in T1 times between normal myocardium and fibrosed myocardium with increased ECV can be accomplished by the administration of a paramagnetic contrast, and in cardiovascular magnetic resonance imaging, gadolinium contrast is used. Two physicians, equally well-educated, will find it easier to agree (i.e., have lower interpersonal discordance) on an image where the fibrosed tissue stands out as "white on black" instead of on an image where fibrosed tissue is light gray than a normally slightly darker gray normal myocardium. Gadolinium is the most often used artificial contrast agent in cardiac magnetic resonance imaging. Gadolinium is administered in a chelated form and is contraindicated in patients with poor kidney function (e-GFR <30 ml/min/1.73 m^2). Gadolinium is effectively a "T1 shortener," and important information can be obtained if the intravascular and intramyocardial behavior of gadolinium is taken into account. Within 8–10 min after intravenous administration of gadolinium, it will have washed through the normal myocardium, but will have diffused into the extracellular spatium of the myocardium and will take longer to diffuse out of this extracellular space again. Chelated gadolinium accumulates in and is slower to wash out of fibrotic areas, and fibrotic areas will therefore appear with a substantially shorter T1 time than normal myocardium after gadolinium administration. Often color-encoded T1 maps are generated in which the pixel (a square in the image) values represent the T1 in each voxel (a cube in the myocardium) rather than a signal intensity in arbitrary units. T1 maps can depict even relatively small variations of T1 within the heart muscle and thereby highlight tissue pathology. T1 mapping without

administration of gadolinium ("native" T1 mapping) is sensitive to myocardial edema, iron overload, and the presence of myocardial infarcts and scarring, but overall the significantly shortened T1 signal associated with gadolinium can be used to quantitate and image the extracellular space.

Myocardial Blood Flow Determination with CMR

If the first pass of gadolinium through the heart and myocardium is imaged with ultrafast MRI sequences, the temporary enhancement offered by gadolinium can be used to determine myocardial blood flow (Sørensen et al. 2020a, b). It is usually performed during rest and during pharmacological vasodilatation with adenosine (Fig. 2). On par with CMR, myocardial blood flow can also be assessed in DM with, for example, positron emission tomography, but CMR has the distinct advantage to other techniques in DM that it will in the same scan precisely quantitate two of the theoretical most prominent factors underlying diabetic cardiomyopathy: myocardial blood flow and myocardial fibrosis. Myocardial blood flow determination with CMR is considered a noninvasive reference standard on par with PET. Since CMR in the same scan quantifies these, and also heart and vascular function, a comprehensive CMR scan will in DM patients quantitate most relevant parameters for noninvasive evaluation of cardiovascular function.

Determination of the Extracellular Volume

Taking the difference between native and post-contrast T1 mapping into consideration, it is possible to determine the precise percentage of extracellular space of cardiac tissue (Arheden et al. 1999; Flett et al. 2010). This technique is built on the principle that at (near-)equilibrium after an infusion of gadolinium, the gadolinium concentration will be the same in serum, as it is in the extracellular space. Hence, the differences between the naive T1 signal (reflecting both intracellular and extracellular myocardial tissues (and blood)) and the post-contrast T1 signal (reflecting mainly extracellular myocardial tissue (and serum) since as said gadolinium is (temporarily) pooled extracellularly) will reflect the relative expansion of the ECV. The percentage of myocardial tissue taken up by the ECV can therefore be determined if only the hematocrit is known. This technique has the advantage that any systematic errors affecting the precise T1 signal should affect T1 mapping of myocardial tissue and blood to the same degree and hence level out such differences.

The Late Gadolinium Enhancement Technique

LGE technique images are produced from the relative difference between normal (myocardium without gadolinium accumulation) and fibrotic (myocardial tissue with excessive gadolinium concentration) areas by choosing a specific time point of imaging

where the normal myocardial will not show a signal whereas the LGE hyperenhanced areas will appear much brighter (usually such images will show fibrotic area as white areas or islands in the normal black myocardium) (Figs. 1 and 2). The precise time point

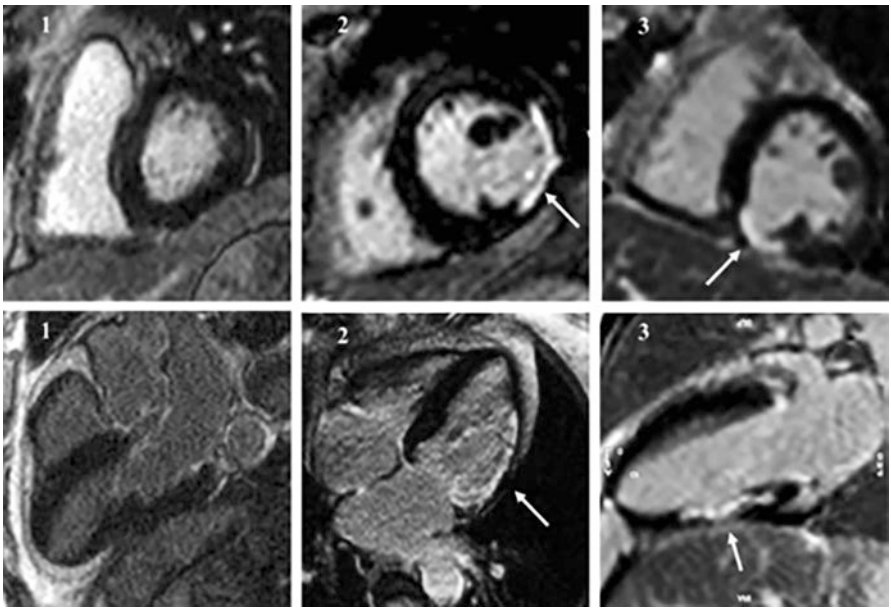

Fig. 1 One normal subject [1] and patients with typical ischemic late gadolinium hyperenhancement (LGE) lesions from previous myocardial infarcts [2, 3]. LV short-axis and long-axis images from the same patients. Normal myocardium appears black, whereas fibrotic areas appear white (white arrows). Ischemic lesions subendocardial/transmural, and in the area of ischemic LGE, there is thinning of the myocardium. (From Bojer et al. (2020a) (open access))

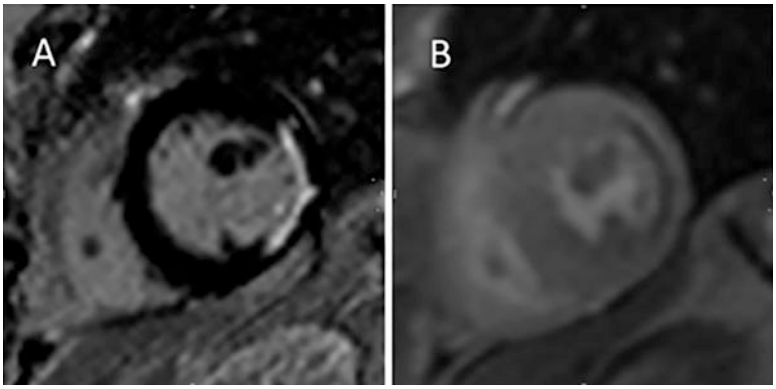

Fig. 2 Subendocardial scar visualized by late gadolinium enhancement (**a**) and coherent subendocardial perfusion defect visualized on perfusion sequence (**b**). (From Sørensen et al. (2020a) (open access))

after which an additional impulse is added after the initial is chosen from images produced with different repetition times based on the original Look-Locker sequence to make certain that at this repetition time, the signal from normal myocardium is "nulled" (i.e., does not display a signal). The difference hence mainly relates to the normal myocardium in LGE images, where often an abundant amount of fibrosis can be found that does not appear white in the white-on-black LGE images. This is visually pleasing to the eye and easily produces robust images of clinical importance. The T1 mapping images are actually equivalent, but now the nulled (black) normal tissue will demonstrate and quantitate the "shades of gray" (or whichever color coding is chosen).

CMR Determination of Fibrosis in Cardiomyopathies

LGE is now a reference standard for noninvasive imaging of myocardial scar and focal fibrosis in both ischemic and non-ischemic cardiomyopathies and makes up for a large fraction of clinical cases in clinical cardiac MRI. The method has a particular value in the differential diagnosis of ischemic versus non-ischemic cardiomyopathy based on the location and transmural extent of scar. Based upon specific LGE patterns in combination with phenotypic determination from film images, some of the non-ischemic cardiomyopathies can be further differentiated. This has recently become of particular interest in patients with DM since these patients often not only have concomitant IHD but have also recently been demonstrated to sometimes display quite specific non-ischemic lesions of importance for heart function not generally seen in other non-ischemic cardiomyopathies. DM is complex in the sense that in most patients, the cardiomyopathy is non-ischemic, i.e., not explained by significant underlying ischemic disease, but on the other hand DM is well-known to be associated with IHD and hence a significant number of patients will show both. LGE change as determined with CMR was early shown to precisely delineate histopathological changes in acute myocardial infarction, and has been demonstrated to reflect prognosis (Schelbert et al. 2015).

Ischemic Versus Non-ischemic Cardiomyopathy with LGE

In ischemic cardiomyopathy perfusion is significantly lowered and usually so in a pattern reflecting previous myocardial infarctions. With an abrupt cessation of the perfusion of a coronary artery, necrosis will initially manifest in the subendocardial part of the affected myocardial segment as a small LGE hyperenhancement. If the coronary artery is not re-perfused (with thrombolysis or percutaneous transluminal angioplasty), thereafter the subendocardial LGE hyperenhancement will spread laterally, and thereafter it will progress in a transmural fashion (Figs. 1 and 2). With time the segment will become akinetic, the LGE will become transmural, and the segment will in line with later fibrotic contraction become thinned. With dilated non-ischemic cardiomyopathy, often the mid-myocardial layers will become fibrosed with thin longitudinally running LGE changes. In hypertrophic

cardiomyopathy LGE changes can often be seen in the hypertrophied areas associated with disarrayed myocytes. In all these cardiomyopathies, the finding of LGE is associated with worse prognosis.

Extracellular Volume and Fibrosis in Diabetes by CMR

Myocardial scars (replacement fibrosis) have previously been documented in DM patients referred to CMR for clinical indications, but systematic studies on cohorts of unselected DM patients are still scarce, and findings of fibrotic areas in DM patients have been suggested to reflect silent coronary events (Kwong et al. 2008; Turkbey et al. 2011). Building on the knowledge from animal and previous pathophysiological studies in humans, DM, even if not significantly associated with ischemic heart disease, may be associated with lowered myocardial blood flow, significant diffuse fibrosis, and perhaps even islands of myocardial fibrosis not related to previous infarcts. CMR studies have now been undertaken to more precisely document LGE and myocardial blood flow changes in larger patient cohorts with T2DM not necessarily documented with previous cardiac disease. Zeng et al. (2017) in rabbits made diabetic with alloxan documented that T1 mapping quantitated induced myocardial diffuse fibrosis in DM well. Ng et al. (2012) documented that patients with T2DM may have diffuse myocardial fibrosis as evidenced with the T1 mapping technique. Consequently, imaging is now suggested to increase in patients with obesity and DM (diabesity) to help combat the increased burden of these (Ng et al. 2021).

Cardiac Fibrosis and Extracellular Volume in Patients with T2DM

One way to document the importance of fibrosis and the associated rarefaction of myocardial smaller vessels with subsequent myocardial hypoperfusion will be to CMR scan larger cohorts of patients with DM and follow them up in order to see the relative impact of the two on cardiac function and outcome. Bojer et al. (2020a) in a 264-pt. strong cohort of nonselected patients with T2DM as the first documented specific non-ischemic lesions with CMR. The cohort had increased echocardiographic markers of impaired LV relaxation but not yet dilated left atrium, and no patient was documented to have HF. 78.4% of these unselected T2DM patients had no LGE lesions, and 11.0% had ischemic LGE lesions (only approximately half of these had previously been suspected of ischemic heart disease) (Fig. 2). Of interest, however, a number of patients (9.5%) had LGE hyperenhancement lesions that were not subendocardial or transmural and hence could not be attributed to previous infarcts (1.1% of patients had both previous infarcts and non-ischemic lesions) (Fig. 3). Such non-ischemic LGE lesions were usually found in the mid-myocardium of the inferolateral basal segments of the LV. The LGE lesions were not as dense as the thoroughly fibrotic subendocardial lesions associated with previous myocardial infarction, and were associated with neither hypokinesis nor

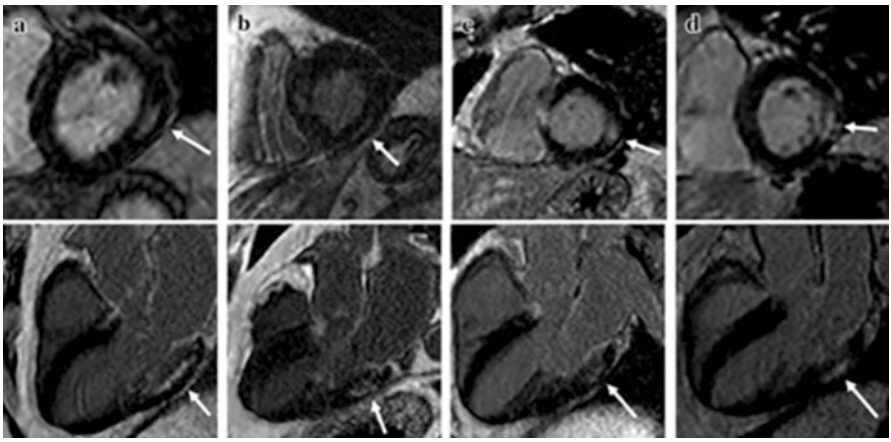

Fig. 3 Four DM2 patients (**a**–**d**) with typical non-ischemic late gadolinium hyperenhancement (LGE) lesions with LV short-axis and long-axis images. Non-ischemic lesions are located mid-myocardial, basal and lateral, or inferolateral. In segments with non-ischemic LGE lesions, the myocardium remains thick. (From Bojer et al. (2020a) (open access))

thinning of the myocardial segment in question. With T1 mapping technique, it was documented that expansion of the ECV was often substantial in patients with T2DM (Bojer et al. 2020a; Sørensen et al. 2020a). In an age- and gender-matched cohort without DM, the ECV was 26.1 (SD 1.5) %, whereas as a group it was significantly higher in T2DM without LGE non-ischemic lesions at 28.8 (SD 2.7) % and even higher in patients with T2DM and LGE non-ischemic lesion at 30.4 (SD 3.1) % (Sørensen et al. 2020). The range was wide, and patients with concomitant IHD had ECV of 32.2 (SD 3.8) % as some patients could be found with ECV above 40% of the total myocardium.

Relation of Cardiac Fibrosis to Markers of Diastolic Function

In comparison with patients without LGE lesions, patients with non-ischemic LGE lesions had increased myocardial mass (150 ± 34 vs. 133 ± 33 g; $P = 0.02$), increased left atrial volume, and increased biomarkers of myocardial stress (NT-proBNP (8.9 (5.9–19.7) vs. 5.9 (5.9–10.1) μmol/L; $P = 0.02$) and high-sensitive troponin (15.6 (13.0–26.1) vs. 13.0 (13.0–14.6) ng/L; $P = 0.007$)) (Bojer et al. 2020a). Whereas diabetic cardiomyopathy firstly and in most patients probably only affect diastolic measures of the heart, Bojer et al. (2020b) recently demonstrated that patients with an extracellular volume in the highest quartile (>31.4% of the myocardium) had a slightly lowered LVEF of 60 (SD 10) % in comparison with all patients with normal or less increased extracellular volume of 64 (SD 7) %. In line with most animal experiments where fibrosis has been seen, the fibrosis of the T2DM patients was significantly associated with echocardiographic signs of diastolic dysfunction (average E/e' at 9.9 (8.7–12.6) vs. 8.8 (7.4–10.7) in patients without;

$P = 0.04$) (Bojer et al. 2020a). The patients with non-ischemic LGE lesions had higher prevalence of retinopathy (48 vs. 25%, $P = 0.009$) and autonomic neuropathy (52 vs. 30.5%, $P = 0.005$) than the patients without such lesions. Taking the well-performed animal and human autopsy studies into consideration, it is likely that this expansion of the ECV is related to the widespread combination of AEGs and fibrosis found in the myocardium of patients with DM. Further, it is probably so that the high ECV demonstrable with T1 mapping technique will eventually become so manifest as to be viewed as localized "islands of fibrosis" with the LGE technique.

Myocardial Blood Flow in T2DM

In the same cohort, Sørensen et al. (2020a, b, c) quantified myocardial blood flow with CMR, and myocardial blood flow was documented to be often severely lowered in DM. With precise CMR quantification of blood flow, blood flow is quantified both at rest and with maximal coronary vasodilatation (accomplished with intravenous adenosine; 140 microg/kg/min). Sørensen et al. (2020a, b, c) showed that in T2DM, the resting blood flow is elevated at 0.81 (SD 0.19) mL/min/g vs. 0.63 (SD 0.12) mL/min/g in normal age- and gender-matched subjects. On the other hand, the myocardial blood flow could be maximally increased with adenosine to only 2.41 (SD 0.90) mL/min/g in T2DM vs. 3.11 (SD 0.81) mL/min/g in normal subjects. The increased resting myocardial blood flow probably largely reflects the larger resting oxygen need of the heart in DM (where heart rate and systolic arterial blood pressure are higher). Thus, the possibility in T2DM to increase myocardial blood flow with stress is significantly hampered as reflected by a myocardial stress index (the ratio between maximally attainable and resting myocardial blood flow) of 5.1 (SD 1.5) in normal subjects but only 3.0 (SD 1.2) in patients with T2DM. Albuminuria and retinopathy were associated with reduced myocardial blood flow during stress in a multiple regression analysis (Fig. 4).

The Relation of Fibrosis to Myocardial Blood Flow

Of interest the maximally attainable myocardial blood flow and ECV covary inversely (R2 of 0.37; Fig. 5); i.e., the higher the extracellular volume fraction (the more fibrosis and AEGs), the lower the maximally attainable myocardial blood flow. Any discussion on causality is obviously made difficult by the fact that this is a cross-sectional study, and hence this covariation could in theory be seen with fibrosis leading to low myocardial blood flow, or low blood flow leading to abundant fibrosis, – or from T2DM being associated with more common factors influencing both to the same degree. Here the animal studies may be of some help. On the one hand, since histology studies have documented that fibrosis in T2DM is often centered around smaller myocardial arteries, it is likely that it is the "fibrotic sheets of the vessels" that limit the maximally attainable myocardial blood flow. On the other hand, it is equally likely that relative ischemia in situations of myocardial stress will contribute to even more

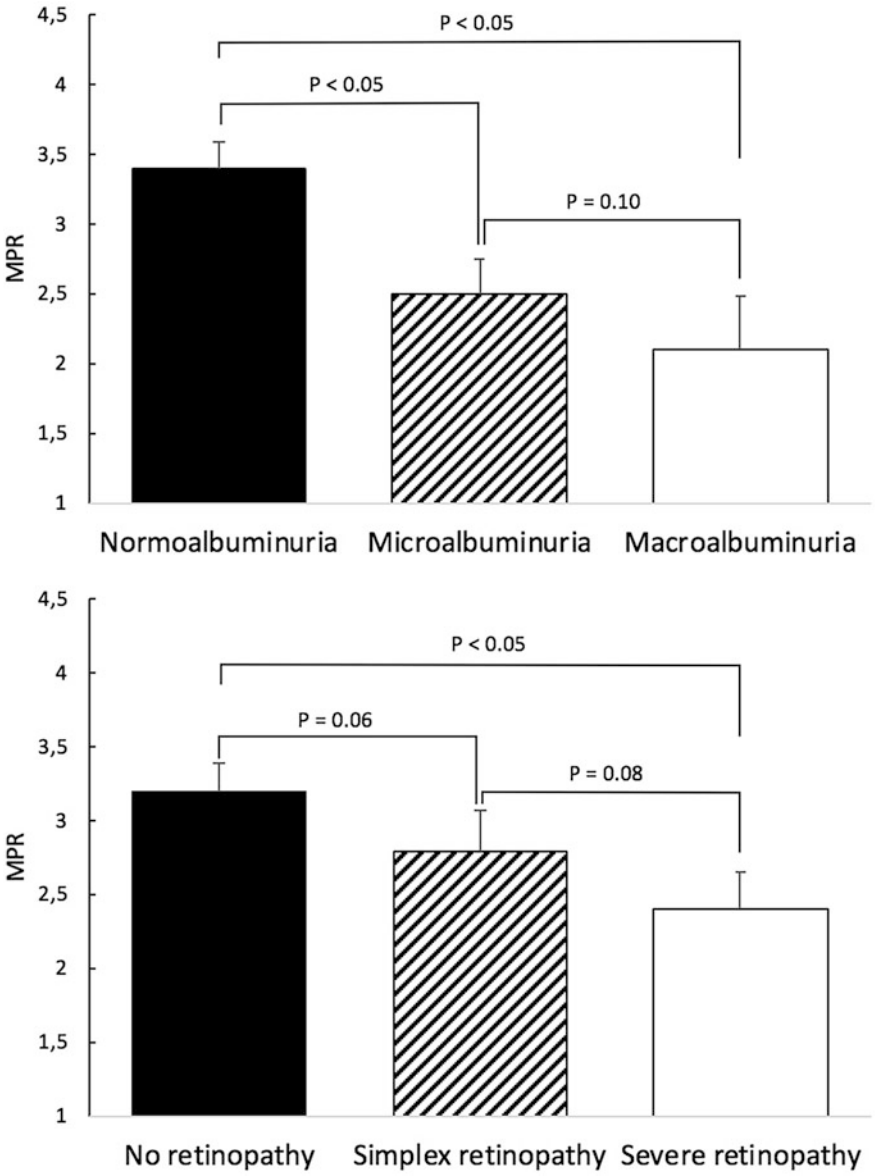

Fig. 4 Mean myocardial perfusion indexes with 95% confidence intervals according to the degree of albuminuria (top) and retinopathy (bottom) in 193 patients with T2DM and 25 normal age- and gender-matched controls. (From Sørensen et al. (2020a) (open access))

fibrosis. Both an increased ECV and myocardial hypoperfusion were associated with diastolic dysfunction, but as documented from animal studies, this may also have been from associated problems, for example, induction of the stiff isoform of titin. It is

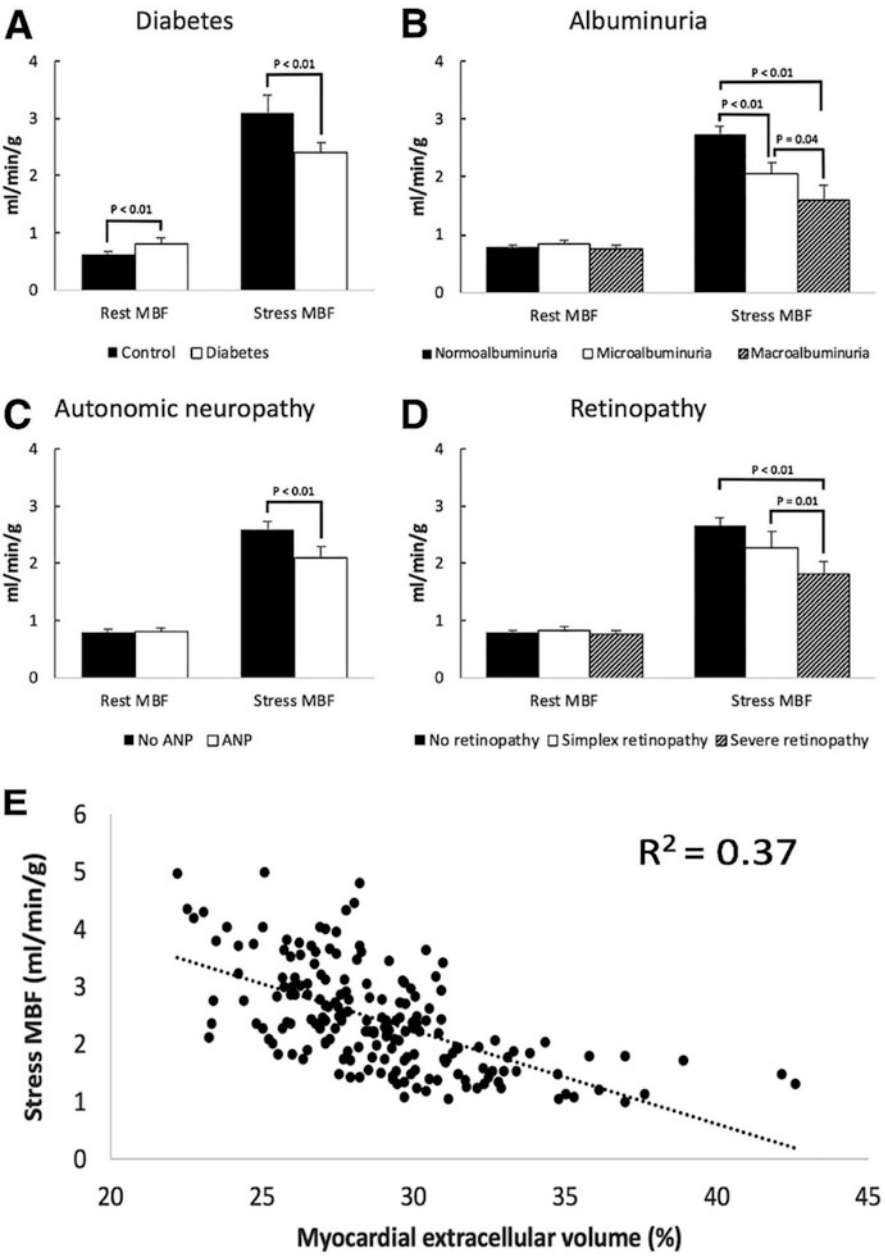

Fig. 5 (**a**) MBF at rest and during stress in patients with T2DM and in control subjects. (**b**–**d**): MBF at rest and during stress in patients with type 2 diabetes with and without various diabetes complications: albuminuria (**b**), autonomic neuropathy (ANP) (**c**), and retinopathy (**d**). (**e**) Correlation between MBF during stress and myocardial ECV in patients with T2DM. (From Sørensen et al. (2020c) (open access))

likely, however, that addressing both myocardial hypoperfusion and expansion of the ECV will prove of value for improvement of diastolic problems. Thus, ischemia (that will be seen with myocardial stress once the maximally attainable myocardial blood flow is lowered significantly) is well-documented to influence the early (adenosine triphosphate-related) unwinding of the LV, and fibrosis must be expected to limit distension of the LV during diastasis.

Looking to the Future

Longitudinal Studies

With the present CMR data on notably myocardial blood flow and myocardial fibrosis and knowing full well from animal studies and pathology and mechanistic studies in humans how this should affect cardiac function, it is now important to establish their relative and precise importance for longer-term outcome in T2DM. Will myocardial hypoperfusion or fibrosis, or both, be of importance for long-term outcome in T2DM, or will they add little to prognosis besides what is already well-established from, for example, echocardiographic measures of diastolic dysfunction, myocardial hypertrophy, and measures of LV afterload (arterial blood pressure and conductance artery distensibility; Kyhl et al. 2021)? Having demonstrated the significant myocardial hypoperfusion seen in T2DM patients and the often significantly elevated extracellular volumes seen, it will now be important to conduct follow-up studies of these patients. It would be important to ascertain if increased fibrosis or lowered myocardial blood flow (or both) should be addressed in intervention studies.

CMR for Screening of DM Patients?

The possibility of CMR with determination of myocardial blood flow and fibrosis is shown to be of value for prediction of cardiovascular outcome; CMR scans may be considered for screening of patients with T2DM in the same way that such patients are routinely evaluated with respect to, for example, eye and kidney function. In this respect it would be important to link imaging findings to interventions. In this respect GLP1 receptor agonists and SGLT2 antagonists have attracted attention and are being evaluated in randomized CMR studies. Recently Bojer et al. (2021b) in a double-blind randomized CMR study documented that weight loss induced by the GLP1 receptor agonist liraglutide did not improve peak filling of the LV (a CMR parameter of LV filling; Bojer et al. 2021a) and hence cannot in the short term be expected to improve cardiac function in T2DM. Improvement of cardiac function – also in the short term – now seems of considerable higher interest with the SGLT2 antagonists that in randomized studies have proven of value for HF treatment in patients both with and without DM (Anker et al. 2021). Randomized mechanistic CMR studies are now ongoing to understand the not yet well-understood but apparently beneficial importance of increasing glucose excretion by the kidneys.

Validation of Serum Biomarkers of Fibrosis

Serum biomarkers of cardiac dysfunction have long been sought, since they obviously offer an immediate window to cardiac problems measured with few problems and potentially with a high degree of precision. The greatest success in biomarkers have been with serum biomarkers of myocardial necrosis, notably CKMB and troponin. Other biomarkers are used to reflect cardiac dysfunction, notably atrial and brain natriuretic peptides. Atrial natriuretic peptide is released from atria in response to increased atrial volume, and brain natriuretic peptide reflects ventricular dysfunction. Atrial natriuretic peptide is mainly used in research, whereas brain natriuretic peptide has been well-established to reflect cardiac dysfunction. Notably, however, brain natriuretic peptide is low in patients with diabesity probably related to the increased dispersion volume of obesity. Hence, in T2DM brain natriuretic peptide will increase in response to overt heart failure, but may not be expected to adequately reflect subclinical cardiac dysfunction. Biomarkers reflecting fibrosis and increased fibroblast activation are now vigorously sought to potentially determine established or ongoing fibrosis formation of the heart, and in patients with T2DM recently Sørensen et al. (2020b) linked a biomarker of fibroblast activation (fibroblast growth factor 23) to impaired cardiac diastolic function and lowered myocardial blood flow during adenosine stress (Fig. 6).

For serum biomarkers of myocardial small vessel disease and myocardial fibrosis, it is a fundamental problem that biomarkers under evaluation must still be expected to suffer from being biomarkers of, for example, all body fibrosis. Hence, increased values, currently being evaluated up against CMR-determined fibrosis, may reflect more widespread organ fibrosis and perhaps not only myocardial. Future studies will, if initial studies are confirmative of the possibility to reflect organ fibrosis by blood-borne biomarkers of fibrosis, be needed to seek out biomarkers with more specificity against the myocardium.

Applications to Prognosis

In this study the technique of magnetic resonance imaging of myocardial blood flow and myocardial fibrosis in diabetes mellitus has been reviewed. Animal studies and pathology studies and previous mechanistic studies in humans have demonstrated that myocardial hypoperfusion (not necessarily related to ischemic heart disease, i.e., atheromatosis of the coronary arteries) and myocardial fibrosis are important and prognostically important aspects of "diabetic cardiomyopathy" (stiff and thickened heart with poor diastolic function). Cross-sectional studies with magnetic resonance imaging have documented that myocardial hypoperfusion and myocardial fibrosis are indeed prevalent (and correlated) in patients with type 2 diabetes mellitus and that both affect diastolic function of the heart. These magnetic resonance imaging biomarkers are now applied in investigating prognosis in type 2 diabetes patients.

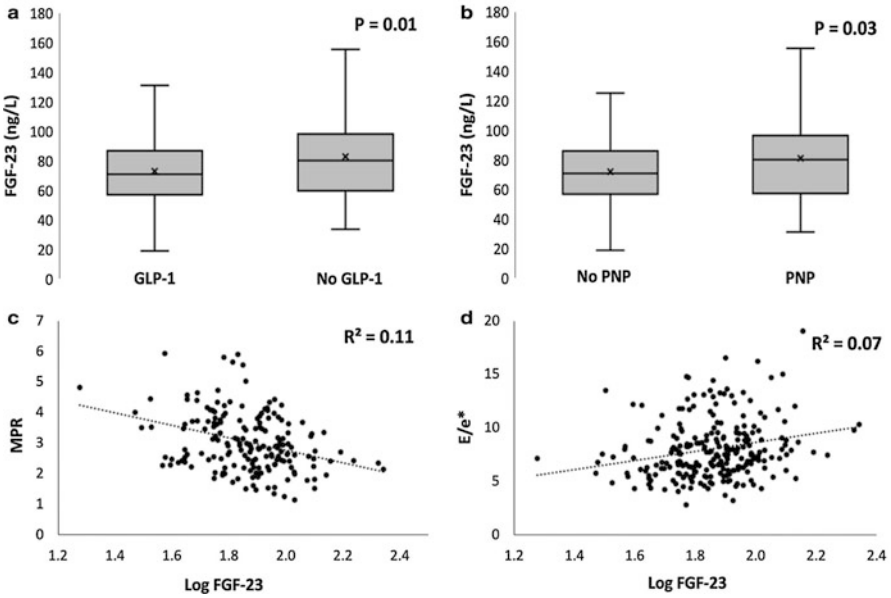

Fig. 6 Fibroblast growth factor 23 in 193 patients with T2DM receiving treatment with GLP1 analogues alone or in combination with other anti-diabetes medication compared to those who did not receive treatment with GLP1 analogues (**a**). Fibroblast growth factor 23 in patients with and without peripheral neuropathy (**b**). Correlation between fibroblast growth factor and myocardial perfusion reserve (MPR) (**c**) and fibroblast growth factor and E/e* as a measure of cardiac diastolic function (**d**). (From Sørensen et al. (2020c) (open access))

Key Facts of Diabetic Cardiomyopathy

- *Diabetes mellitus is associated with a high number of different organ dysfunction, but the significantly lowered life span of a patient with diabetes is related to heart disease.*
- *Diabetes mellitus is associated not only with ischemic heart disease but also with a variant of a non-ischemic cardiomyopathy (*i.e., *not related to atherosclerosis of the coronary arteries or indeed previous myocardial infarctions) known as "diabetic cardiomyopathy."*
- *Features of diabetic cardiomyopathy include cardiac hypertrophy and diastolic dysfunction (cardiac dysfunction with impaired ease of filling of the LV and consequent increased end-diastolic pressure) but not necessarily systolic dysfunction.*
- *Diabetes patients with signs of heart failure would therefore often be described as having "heart failure with preserved ejection fraction," the precise underlying cause of which is not well-described.*
- *The cardiac hypertrophy in diabetes is related to the often accompanying arterial hypertension, but the profound metabolic derangement associated with type 2 diabetes mellitus may set the scene for a large range of myocardial changes.*
- *Animal studies describe vascular rarefaction and widespread fibrosis in the diabetic heart.*

Key Facts of Cardiac Magnetic Resonance Imaging of Fibrosis

- *Cardiovascular magnetic resonance imaging is a noninvasive technique with few contraindications and is therefore suitable for examination of cardiac function in patients with diabetes.*
- *With magnetic resonance imaging, cardiac function (including systolic and diastolic function) can be determined, and with the use of chelated gadolinium for contrast, myocardial blood flow and myocardial fibrosis can be determined, all in the same scan.*
- *Magnetic resonance imaging of myocardial blood flow and quantification of myocardial fibrosis are built on T1-weighted sequences. T1 is the time after with protons ("flipped" in the static magnetic field of the MR scanner by a radio-frequency pulse) have equilibrated 63%.*
- *The T1 time of a tissue in question will be influenced by its amount of fibrosis. The difference between normal and fibrotic myocardium can be maximized by the use of chelated gadolinium for contrast since chelated gadolinium (with a proper selection of scan time) will temporarily accumulate in fibrotic tissue and since gadolinium will significantly lower the T1 time.*
- *With magnetic resonance imaging, population studies can be performed documenting not only cardiac function but also quantitative measures of myocardial blood flow and fibrosis.*
- *Magnetic resonance imaging has now been used to document significantly lowered myocardial blood flow in patients with type 2 diabetes mellitus. In an unselected cohort of patients with type 2 diabetes, the perfusion index (ratio between the resting value and the maximally attainable value) will be lowered to approximately 3, whereas it is usually approximately 5.*
- *Magnetic resonance imaging has now documented significant diffuse fibrosis in a number of patients and in some patients (the patients with the highest values for extracellular volume (fibrosis) virtual islands of fibrosis.*
- *Such islands of fibrosis, not related to previous infarcts, are usually found in the mid-myocardium of the inferolateral and basal parts of the left ventricle, and are associated with particularly poor left ventricle diastolic dysfunction and probably represent myocardial areas of fibrosis previously described during autopsy studies in patients with diabetes mellitus, who had died from congestive heart disease but without signs of significant ischemic heart disease.*

Mini-Dictionary of Terms

- **Gadolinium** – The most often used artificial contrast used for cardiovascular magnetic resonance imaging studies. Gadolinium is effectively a "T1 shortener," and vessels or myocardium containing gadolinium will in appropriate images therefore appear bright ("hyperenhanced").
- **Heart failure with preserved ejection fraction** – Heart failure with a preserved ejection fraction, i.e., heart failure symptoms despite an LVEF close to the normal of at least 55%.

- **Heart failure with reduced ejection fraction** – Heart failure with an impaired left ventricle ejection fraction below 40% (normally above 55%).
- **Myocardial blood flow** – The amount of blood traversing the heart muscle per minute per 100 g. The myocardial blood flow is determined from mathematical (Fermi) deconvolution of the myocardial first-pass perfusion signal by gadolinium contrast.
- **Myocardial extracellular volume** – The volume of the myocardium not taken up by cardiomyocytes. It is normally around 26 (SD 1.5) % but may in T2DM increase >40% secondary to expansion by collagen, glycated hemoglobins, and water.
- **Myocardial fibrosis** – Collagen in combination with glycated hemoglobin and water expanding the myocardial extracellular volume.

Summary Points

- *Diabetes mellitus (both type 1 and type 2) is significantly associated with cardiomyopathy, which can be ischemic or non-ischemic.*
- *The non-ischemic variant of cardiomyopathy (known as "diabetic cardiomyopathy") is related to myocardial hypertrophy and vascular rarefaction with expansion of the extracellular volume by glycated hemoglobin and widespread diffuse fibrosis.*
- *Patients with DM and signs of cardiomyopathy are usually examined with echocardiography and possibly coronary CT/coronary angiography if significant coronary atheromatosis is suspected, but such techniques will not document the known underlying pathogenetic factors of myocardial hypoperfusion and myocardial fibrosis.*
- *With magnetic resonance imaging using gadolinium contrast, it is possible to noninvasively quantitate myocardial blood flow and myocardial fibrosis in the same study and obtain precise knowledge on cardiac and vascular function.*
- *In a cohort of patients with type 2 DM, resting myocardial blood flow is elevated (reflecting the increased pulse-pressure product of patients with DM), whereas the maximally attainable myocardial blood flow (with adenosine) is significantly lowered.*
- *In patients with T2DM, the vascular rarefaction results in an ability to increase myocardial blood flow only threefold during stress, whereas it is normally fivefold.*
- *In normal subjects as determined with T1 mapping technique, 25% of the myocardium is taken up by the extracellular volume, but in T2DM patients this fraction will increase, in a few patients to values >40%.*
- *In some patients with high extracellular volumes, virtual islands of fibrosis can be detected with the late gadolinium contrast technique.*
- *These areas probably reflect the fibrotic islands initially described by Rubler* et al. (1972) *in the pathology studies initially describing diabetic cardiomyopathy.*

- *Follow-up studies are now pursued in order to determine the relative importance of myocardial hypoperfusion and myocardial fibrosis in the prognosis of patients with T2DM.*

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Measures of Endothelial Function in Type 2 Diabetes: A Focus on Non-circulatory Methods of Measurement

41

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Contents

Introduction 850
Noncirculatory Measures of Endothelial Function 851
Venous Occlusion Plethysmography (VOP) 852
Flow-Mediated Dilatation 854
Peripheral Artery Tonometry (PAT) 856
Aortic Pulse Wave Velocity (PWV) 856
Laser Doppler Flowmetry (LDF) 859
Skin Autofluorescence (SAF) 860
Applications to Diabetes 860
Mini-dictionary of Terms 861
Key Facts of Endothelial Dysfunction in Type 2 Diabetes Mellitus 861
Summary Points 861
References 862

Abstract

The endothelium is a vital organ important for regulating vascular homeostasis. Endothelial dysfunction in type 2 diabetes has been linked to the development of micro- and macrovascular complications. In particular, its association with the onset of cardiovascular events has prompted the development of numerous methodologies to evaluate endothelial function. This chapter describes the non-circulatory methods of measurement of endothelial dysfunction, such as venous occlusion plethysmography, flow-mediated dilatation, peripheral artery tonometry, aortic pulse wave velocity, and laser Doppler flowmetry. We discuss their applicability and evidence for use in T2DM. Flow-mediated dilatation and pulse wave velocity are currently among the most widely used and validated techniques for cardiovascular risk stratification in patients with T2DM, as they correlate with

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glycemic control and cardiovascular risk factors. Main disadvantages of these vasomotor techniques are the need for a skilled technician, operating time, and physiological variation, limiting them currently to the research setting.

Keywords

Endothelial dysfunction · Diabetes · Sheer stress · Vasomotor function · Cardiovascular events · Flow-mediated dilatation · Peripheral artery tonometry · Reactive hyperemia · Pulse wave velocity · Augmentation index · Laser Doppler flowmetry

Abbreviations

AGEs	Advanced glycation end products
AIx	Augmentation index
AP	Augmentation pressure
BMI	Body mass index
cfPWV	Carotid-femoral pulse wave velocity
EPC	Endothelial progenitor cell
ESRF	End-stage renal failure
FMD	Flow-mediated dilatation
LDF	Laser Doppler flowmetry
MRI	Magnetic resonance imaging
NO	Nitric oxide
PAT	Peripheral artery tonometry
PP	Pulse pressure
PU	Perfusion unit
PWA	Pulse wave amplitude
PWV	Pulse wave velocity
RHI	Reactive hyperemia index
SAF	Skin autofluoresescence
SGLT2	Sodium-glucose cotransporter-2
T2DM	Type 2 diabetes mellitus
VOP	Venous occlusion plethysmography

Introduction

The vascular endothelium plays an important role in maintenance of vascular homeostasis, regulating vascular tone, angiogenesis, and coagulation. Endothelial dysfunction is characterized by an impairment of these physiological functions, predisposing to vascular inflammation, atherosclerosis, and end-organ damage (Avogaro et al. 2011).

Endothelial dysfunction is well-recognized to occur in both type 1 and type 2 diabetes mellitus (T2DM), contributing to their micro- and macrovascular complications and multisystemic involvement. The pathogenesis is complex – ranging

Table 1 Characteristics of the ideal technique for measurement of endothelial function

Methodology	Noninvasive Easy to perform Objective Standardized Cost-effective
Results	Reproducible Accurate Valid reference ranges
Utility	Aids prognostication of vascular diseases Adds incremental value over traditional risk factors Reversible with intervention for monitoring of therapy Predicts risk sufficiently to change recommended therapy Improves clinical outcomes

from hyperglycemia-induced oxidative stress, endothelial cell apoptosis, and defective endothelial progenitor cell (EPC) mobilization and survival (Fiorentino et al. 2013; McClung et al. 2005; Menegazzo et al. 2012). Present at an early stage of T2DM, endothelial dysfunction has also been observed with prediabetes and the insulin-resistant state preceding the onset of diabetes and development of frank hyperglycemia (Wasserman et al. 2018). In contrast, younger adults with type 1 diabetes have preserved endothelial function till much later on in the progression of disease (Heier et al. 2018).

Endothelial dysfunction is characterized by a reduced bioavailability of vasodilators and an increase in endothelium-derived contraction factors, resulting in a loss of endothelium-mediated vaso-relaxation in response to sheer stress and pharmacodynamic stimulation (Bonetti et al. 2003). This chapter aims to describe the noncirculatory methods of measurement of endothelial function and discuss their clinical utility in T2DM. An ideal technique for measurement of endothelial function should be noninvasive, reproducible, accurate, easy to perform, standardized, cost-effective, can be objectively measured, have valid reference ranges, correlates with the prognosis of vascular diseases, improves risk stratification above current traditional risk factors, is reversible with intervention, predicts risk sufficiently to change recommended therapy, and improves clinical outcomes (Table 1) (Flammer et al. 2012; Vlachopoulos et al. 2015). As such, the ideal technique should be able to guide the clinician toward adequate primary or secondary prevention of vascular disease.

Noncirculatory Measures of Endothelial Function

Endothelium-dependent vasomotion is a widely used method for assessment of endothelial function. An impaired vasomotor response was first demonstrated by Ludmer et al. in 1986 by the demonstration of paradoxical acetylcholine-induced vasoconstriction as measured by coronary angiography in subjects with coronary atherosclerosis (Ludmer et al. 1986). This led to interest in the measurement of

coronary endothelial function as a manifestation of atherosclerosis, by various techniques including the use of intravascular ultrasound and quantitative coronary angiography to image coronary vasoactive reactivity (Puri et al. 2013), as well as the measurement of coronary blood flow during maximal coronary hyperemia with provocative stimuli (Stoller and Seiler 2017; Díez-delhoyo et al. 2015).

These techniques are primarily limited by their invasive nature, rendering them inappropriate in the asymptomatic patient without clinical indication for coronary angiography. Less invasive techniques have since been derived to assess the vascular function in peripheral vascular beds, in particular the forearm circulation, and their use has been extended beyond coronary artery disease (CAD) to the evaluation of a multitude of diseases involving macrovascular and microvascular dysfunction. As the assessment of coronary endothelial function in CAD is not the intended focus of our discussion, more detailed attention will be given to peripheral techniques of assessing endothelial function in T2DM, given its multisystemic involvement. Importantly, these less invasive tests involving the peripheral circulation (usually the brachial artery and forearm circulation) have been found to correspond to the coronary endothelial function and are both predictive of cardiovascular disease (Takase et al. 1998; Takase et al. 2005; Matsuzawa et al. 2015). Table 2 gives a summary of a comparison of noncirculatory measures of endothelial function.

Standardization of environmental conditions and adequate subject preparation are important for the reproducible measures of vasomotor function, since many factors such as food, exercise, caffeine, alcohol, menstrual status, temperature, drugs, and mental stress may affect measurements (Keogh et al. 2005; D'Urzo et al. 2018). Hence, guidelines recommend that subjects should be fasted for >6 h, avoid exercise for >24 h, refrain from alcohol and caffeine for >12 h, and avoid smoking for >6 h (Thijssen et al. 2019). Subjects should be supine and relaxed for >10–15 min in a quiet temperature-controlled room. If drugs are taken, a time off the medication of 4 times the half-life of the drug is recommended (Thijssen et al. 2019), particularly if they are known to interfere with vascular function, for example, nitrates, calcium channel blockers, metformin, and SGLT2 inhibitors (Velmurugan et al. 2016; Oshima et al. 2005; Hamidi Shishavan et al. 2017; Zainordin et al. 2019).

Venous Occlusion Plethysmography (VOP)

One of the oldest methods to study human vascular physiology, VOP, is based on the principle of forearm volume increase when venous outflow from the arm is interrupted while arterial inflow continues. The hands are excluded from the circulation by inflation of a wrist cuff 50 mmHg above systolic blood pressure, as blood flow to the hands has different physiology from forearm blood flow, and comprise of multiple arteriovenous shunts through the skin (Whitney 1953). The study is performed with subjects supine and relaxed at ambient temperature of 22–26 °C in order to keep arterial perfusion pressure constant, with the forearm positioned above the level of the heart. Venous return is occluded by an upper arm cuff which inflates to 40 mmHg (above venous pressure but below diastolic pressure), for ten intervals

Table 2 Comparison of noncirculatory measures of endothelial function

Technique	Vascular bed	Advantages	Disadvantages
Venous occlusion plethysmography	Forearm vasculature	Easy access Well-validated Allows local infusion of vasoactive medications for study of dose-dependency Reproducible if setup is controlled	Semi-invasive Significant inter-subject variability Lack of standardized methodology Time-consuming
Flow-mediated dilatation	Brachial artery	Noninvasive Reproducible Accurate Availability of standardized protocol Many CV outcome studies	Steep operator learning curve Technically challenging Poor reproducibility if not performed in a standardized manner Relatively expensive
Peripheral artery tonometry	Finger microvasculature	Noninvasive Simple to perform Less operator-dependent Automated Reproducible Some CVV outcome studies	Less well-established PAT signal can be influenced by variable nonendothelial-dependent factors Single-use probes required
Pulse wave velocity	Large conduit artery usually aorta	Noninvasive Relatively simple to perform Reproducible Accurate Well-validated Many CV outcome studies	Need to expose groin for cfPWV Many different sites of measurement
Laser doppler flowmetry	Skin microvasculature	Noninvasive Simple	Relatively expensive Sensitive to movement artifacts Steep operator learning curve Not solely indicative of endothelial function, also represents global microvascular response

followed by 5 s of deflation for emptying of forearm veins (Wilkinson and Webb 2001). Strain gauges around the forearm detect changes in forearm volume by a change in electrical resistance in response to arm circumference change. Flow is usually expressed in milliliters per 100 ml volume of forearm per minute (Benjamin et al. 1995) and reflects the contractile tone of the vascular smooth muscle. This is compared to the contralateral arm which acts as a control, in response to local infusion of vasoactive substances into the brachial artery. The technique is still

considered minimally invasive but is generally well tolerated since the infusion of substances takes place some distance away from the coronary vasculature.

After a period of forearm ischemia, reactive hyperemia occurs as demonstrated by a surge in forearm blood flow which then gradually returns to baseline. Forearm blood flow can be used to calculate forearm vascular resistance; however, this assumes laminar blood flow and may be misleading if baseline blood pressure differs between subjects, as blood pressure differences affect smooth muscle contraction. Expressing data as percentage change in ratio of forearm blood flow in infused versus control arms can overcome the challenges of between group comparisons for good reproducibility (Benjamin et al. 1995).

Previously considered the gold standard for the study of vascular endothelial function, VOP is impractical in the clinical setting and has largely been replaced by noninvasive methods such as flow-mediated dilatation (FMD) which is now more widely used.

Flow-Mediated Dilatation

Sheer stress induced by an increase in blood flow after a period of ischemia acts on the endothelium to result in FMD via the release of nitric oxide (NO). FMD is measured by high resolution B-mode ultrasound imaging of the diameter change of a conduit artery, typically the brachial artery (Fig. 1). The brachial artery is occluded by a blood pressure cuff situated above the antecubital fossa which is inflated usually to $\geq$50 mmHg above systolic pressure (or between 200 and 300 mmHg) for 5 min. Its release induces reactive hyperemia, reflected by an increase in blood flow and sheer stress. FMD is expressed as the change in diameter after stimulation at approximately 60–180 s post cuff deflation as a percentage of baseline prior to cuff inflation (Barac et al. 2007; Thijssen et al. 2019). This endothelium-dependent vasodilatation due to sheer stress, largely mediated by nitric oxide, is distinct from endothelium-independent smooth muscle-mediated vasodilatation, for example, induced by sublingual nitroglycerin.

Ideally, a conduit vessel diameter between 2.5–5 mm would give the best accuracy and reproducibility with imaging. Vessels too small have large percentage changes in diameter despite only small absolute measurement changes, overestimating endothelial function, while vessels too large do not dilate significantly even with normal endothelial function. Alternative conduit arteries that can be studied include the femoral and radial arteries if the brachial artery is unsuitable. If lower limb arteries are used, it is important to consider that peak dilatation may occur later than 180 s postcuff deflation (Thijssen et al. 2008). Variations of FMD with the cuff position either distal or proximal to the imaged artery have been used, which were no different in their prognostic value for cardiovascular events (Matsuzawa et al. 2015).

The main advantage of this method is its noninvasive nature. Furthermore, FMD has shown accuracy even at submillimeter readings and is highly reproducible. However, strict experimental conditions need to be standardized to limit within-subject variability. If performed carefully, the overall coefficient of variation is 1.8%,

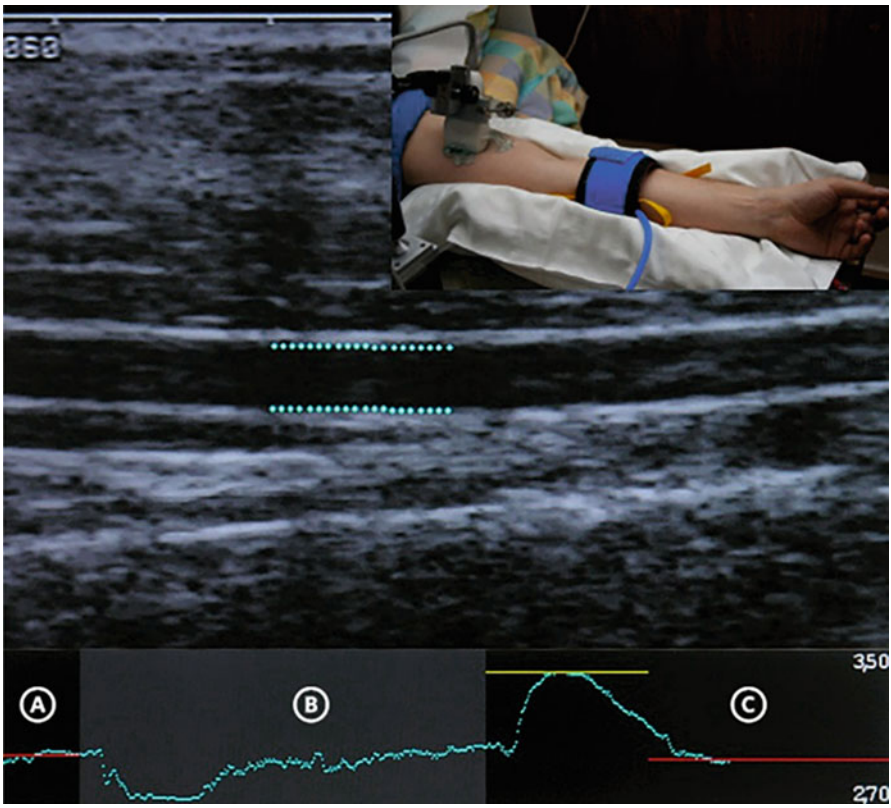

Fig. 1 Flow-mediated dilatation: longitudinal section of the brachial artery in 2D-mode. The graph shows the diameter of the artery at baseline (**a**), during 5 min of cuff inflation (**b**), and vasodilation during reactive hyperemia (**c**). (From "Endothelial dysfunction in cardiovascular disease and Flammer syndrome – similarities and differences" by Barthelmes et al. 2017, licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/))

with significant day-to-day variation but little between weeks or months (Sorensen et al. 1995). Since its development in 1992 (Celermajer et al. 1992), FMD has become the most commonly used method to evaluate vascular endothelial function noninvasively. Practice guidelines have been developed by the American College of Cardiology in 2002 (Corretti et al. 2002) and the European Society of Cardiology in 2019 (Thijssen et al. 2019) for standardization to reduce intraoperator variability.

Another advantage lies in its rapid response with intervention, allowing the study of these pharmacological agents in the treatment of endothelial dysfunction (Charakida et al. 2010). For example, some glucose-lowering agents including metformin, sodium-glucose cotransporter-2 (SGLT2) inhibitors, and thiazolidinediones have shown improvement in endothelial function using FMD (Shigiyama et al. 2017; Stojanović et al. 2016; Romualdi et al. 2008). However, whether these translate to clinical outcomes need to be further evaluated. FMD has been

extensively studied to be associated with established cardiovascular risk factors including diabetes, smoking, and hypertension, as well as existing cardiovascular disease. Meta-analyses have linked impaired brachial FMD with the development of future cardiovascular events (Inaba et al. 2010; Xu et al. 2014). However, the evidence for the incremental value of FMD above estimation by traditional cardiovascular risk factors, for example, the Framingham risk score, is conflicting and has not been well-established (Yeboah et al. 2009; Park et al. 2014; Peters et al. 2012). Furthermore, FMD measurements require significant specialized training; hence, currently its use is primarily confined to the research setting.

Peripheral Artery Tonometry (PAT)

PAT, also known as finger plethysmography, is an alternative noninvasive measure of endothelial function developed in 2003 (Kuvin et al. 2003). Like FMD, PAT is also based on the principal of reactive hyperemia after transient arterial occlusion. The PAT measures the beat-to-beat arterial pulse wave amplitude (PWA) at the fingertip (Fig. 2). A finger probe on the distal index finger applies a uniform pressure of 70 mmHg to avoid venous pooling which can induce reflex vasoconstriction. One probe is sited on the arm undergoing hyperemia testing and another on the contralateral arm. The reactive hyperemia index (RHI) is the average PWA for 1 min occurring 60 s after the onset of reactive hyperemia, divided by the average PWA at baseline preocclusion, and normalized against that from the contralateral arm (Kuvin et al. 2003).

Compared to FMD, PAT is seen to be more simplified, less technically challenging, requiring less training to perform and is less operator-dependent (Kuvin et al. 2003). Like FMD, RHI obtained by PAT correlates with cardiovascular risk factors and is predictive of cardiovascular events (Kuvin et al. 2003; Matsuzawa et al. 2015). However, results on FMD and PAT are not directly interchangeable and appear to measure distinct aspects of cardiovascular risk (Fukumoto et al. 2021; Hamburg et al. 2011). This may reflect the different circulations assessed – FMD measures macrovascular endothelial function of conduit arteries, while PAT examines the microvascular endothelial response of the peripheral resistance arteries. It is hypothesized that different vascular beds of various vessel sizes may have different physiological response to ischemia or differing sensitivity to early damage by specific cardiovascular risk factors, for example, FMD is more sensitive to risk factors such as age, smoking, and hypertension while RHI is more sensitive to body mass index (BMI) and diabetes (Hamburg et al. 2011).

Aortic Pulse Wave Velocity (PWV)

Aortic PWV is widely used as a measure of arterial stiffness, which is an early marker of arterial remodeling (Segers et al. 2020). PWV, expressed in m/s, is determined by dividing the distance between two sites along a vascular segment

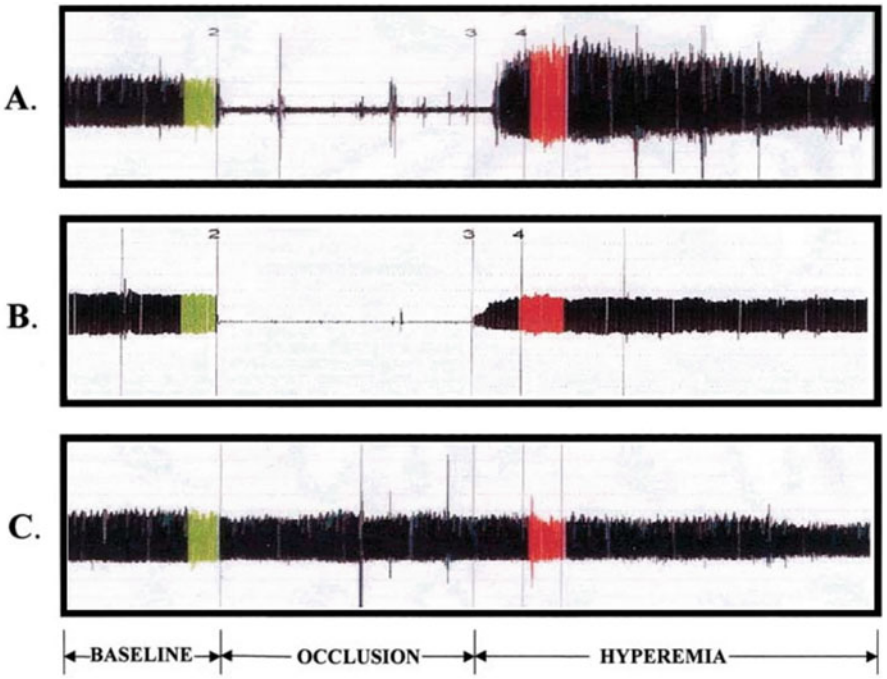

Fig. 2 Peripheral artery tonometry: PWA recordings. PAT at baseline (green), during cuff occlusion and during reactive hyperemia (red): (**a**) Healthy individual with no cardiovascular risk factors showing a steady-state PAT signal, complete disappearance of the signal during cuff inflation, followed by an increased PAT signal during recovery (hyperemia); (**b**) individual with CAD showing a blunted finger PAT response during reactive hyperemia; and (**c**) PAT recording from the contralateral finger not undergoing reactive hyperemia testing in the same patient with CAD. (From "Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude" by Kuvin et al. 2003 with permission)

by the time taken for the pulse waveform to transit between these two sites (speed = distance/time). The higher the PWV, the greater the degree of arterial stiffness, since waves travel faster in a rigid tube with a loss of compliance. Increased PWV has been shown to correlate inversely with endothelial function as measured by FMD (McEniery et al. 2006; Jadhav and Kadam 2005). However, arterial stiffness is not synonymous with endothelial function and limited studies have used PWV to assess endothelial dysfunction. To assess endothelial function, PWV can be combined with pharmacological stimuli such as inhaled salbutamol, with a blunted augmentation index (AIx) response observed in subjects with endothelial dysfunction (Stoner et al. 2012; Wilkinson et al. 2002; Hayward et al. 2002).

AIx is calculated from the proportion of augmentation pressure (AP) divided by the central pulse pressure (PP) (Fig. 3). This represents the proportion of central PP that is attributed to the arterial reflected wave, which is not only a measure of arterial stiffness but also reflects the diameter and elasticity of small arteries and arterioles. This indicates

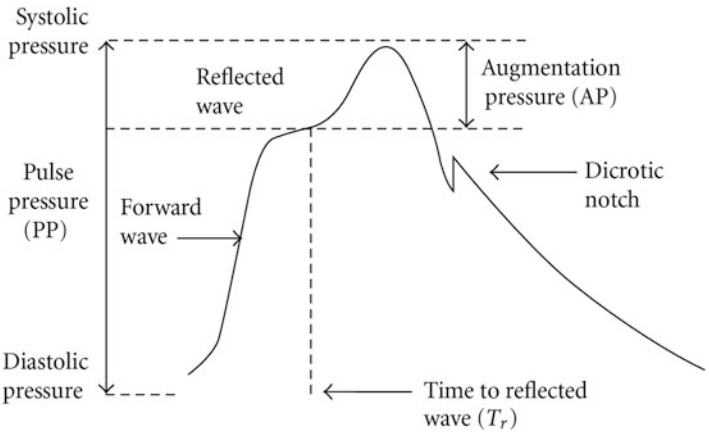

Fig. 3 Aortic pulse pressure waveform. Augmentation index is defined as the augmentation pressure as a percentage of the pulse pressure. (From "Assessments of Arterial Stiffness and Endothelial Function Using Pulse Wave Analysis" by Stoner et al. 2012, licensed under CC BY 3.0 (https://creativecommons.org/licenses/by/3.0/))

abnormalities of the microcirculation including calcification of small arterioles and impaired endothelium-mediated dilatation (Soga et al. 2008). AIx is influenced by vasoactive drugs independently of PWV (Kelly et al. 2001). AIx increases with age and also varies with mean arterial pressure, body height, and heart rate, so these factors should be corrected for prior to interpretation (Stoner et al. 2012). AIx can also be directly measured by applanation tonometry at the common carotid artery, where a greater AIx is a predictor of cardiovascular mortality in end-stage renal failure (ESRF) patients, even in patients with normal PWV (London et al. 2001).

In T2DM however, AIx may be less valuable in predicting cardiovascular outcomes, due to the preferential stiffening of central over peripheral arteries (Kimoto et al. 2003; Wu et al. 2016). As AIx is a ratio of AP to PP, a greater PP due to central stiffening may result in normal AIx in T2DM despite the presence of generalized arterial stiffening and endothelial dysfunction (Zhang et al. 2011).

Various locations for aortic PWV measurements can be obtained, most commonly not only from the carotid to femoral artery (cfPWV), but also branchial-ankle, aortic root-descending artery, ascending aorta-femoral, finger-toe, or heart-femoral arteries. Arterial pulse waveforms can be detected by applanation tonometry, ultrasound doppler, cuff-based oscillometry, or magnetic resonance imaging (MRI) (Cavalcante et al. 2011; Wilkinson et al. 2020). PWV has been shown to be accurate, reproducible, well-validated, and noninvasive (Sutton-Tyrrell et al. 2001). Importantly, aortic PWV has been established to be an independent predictor of cardiovascular events and all-cause mortality (Vlachopoulos et al. 2010; Hametner et al. 2021), with prognostic value above and beyond the traditional Framingham risk score, especially in younger individuals with intermediate cardiovascular risk (Ben-Shlomo et al. 2014; Wilkinson et al. 2020). A PWV reduction of 1 m/s is associated with an adjusted relative risk of 0.71 for all-cause mortality (Guerin et al. 2001). Its ability to

predict cardiovascular events and mortality was also demonstrated in patients with diabetes and glucose intolerance (Cruickshank et al. 2002; Gajdova et al. 2017). In T2DM, increased aortic stiffness is also associated with microvascular complications (Cardoso et al. 2009; Kim et al. 2012).

The simplicity of use and operator-dependence is dependent upon the sites used, for example, carotid-femora is easier to image than ascending aorta-femoral location (Sutton-Tyrrell et al. 2001), and brachial-ankle PWV is simpler than cfPWV despite its limitations (Sugawara and Tanaka 2015). The method is relatively easy to use, with accurate readings easily performed after a short learning period. A methodological consensus has been published for the measurement of cfPWV (Van Bortel et al. 2012), which remains the most well-validated method, with a value >10 m/s representing higher cardiovascular risk in the latest European Society of Cardiology hypertension guidelines (Williams et al. 2018). Despite its evidence for prognostication, the authors conclude that it is not practical enough to be recommended for routine clinical use (Williams et al. 2018).

Laser Doppler Flowmetry (LDF)

LDF is a noninvasive method of assessing alterations in the microvascular function of the skin, which is postulated to reflect systemic vascular dysfunction early in the development of cardiovascular disease (Holowatz et al. 2008). LDF is based on the change in wavelength of light when it is reflected by moving red blood cells in the microvasculature. Measurements are expressed as flux, in perfusion units (PU) or millivolts (1PU = 10 mV). LDF probes can be placed on the medial aspect of the forearm on intact skin.

This can be measured before and after stimulation, for example, by postocclusive hyperemia induced by brief arterial occlusion with a cuff placed on the upper arm. Unlike in other vascular beds, endothelial-derived factors such as nitric oxide and prostaglandins do not play an important vasodilatory role in cutaneous reactive hyperemia, but rather this is dependent on sensory nerves and calcium-activated potassium channels (Lorenzo and Minson 2007; Zhao et al. 2004). Hence, cutaneous reactive hyperemia as measured by LDF may represent a complex microvascular response which may not be entirely endothelium-dependent.

LDF in combination with intradermal administration of acetylcholine by iontophoresis has also been studied, involving the local transfer of charged substances across the skin with a weak electric current to induce endothelium-dependent vasodilatation. This method is said to be better for assessing cutaneous microcirculatory endothelial function than reactive hyperemia (Rossi et al. 2004) and has been found to correlate well with FMD (Debbabi et al. 2010). Local thermal hyperemia has been shown to be impaired in diabetes (Fuchs et al. 2016); however, this may be more reflective of damage to the neurogenic cutaneous vasodilator rather than endothelial dysfunction (Wick et al. 2006; Stansberry et al. 1999).

Currently, clinical use of LDF in diabetes is limited by a lack of measurement standardization, variability in parameters used leading to low reproducibility,

significant intrasubject variability, differences in devices used, need for special data processing software, and a highly qualified operator (Barac et al. 2007; Kulikov et al. 2017). Like other techniques of assessing vasomotor responses, it is also prone to fluctuations in experimental conditions and subject positioning.

Skin Autofluorescence (SAF)

The measurement of cutaneous advanced glycation end products (AGEs) by SAF is a noncirculatory measure of endothelial function that is detailed in the next chapter under AGEs.

Applications to Diabetes

Individuals with diabetes have impaired endothelial function. In the hyperglycemic state, there is impaired metabolic homeostasis that results in chronic inflammation, oxidative stress, procoagulability, and impaired vascular repair, inciting pro-atherosclerotic changes to the vessel wall (Xu and Zou 2009). This is evidenced by reduced vascular reactivity as measured by VOP, FMD, and PAT, and increased arterial stiffness as measured by PWV (Petrofsky et al. 2005; Clarkson et al. 1996; Mori et al. 2019; Elias et al. 2017). FMD and PWV are among the most widely used and validated techniques for cardiovascular risk stratification in patients with T2DM (Villano et al. 2020; Naka et al. 2012; Cruickshank et al. 2002). The disadvantage of FMD and PAT is the need for a skilled technician, limiting these techniques currently to the research setting.

Importantly, cfPWV correlates with glycemic control, with improvement in arterial stiffness observed in individuals who attained better glycemic control, together with improvements in blood pressure and heart rate (Ferreira et al. 2015). In patients with well-controlled T2DM, FMD and serum NO levels were higher, highlighting the importance of glycemic control on endothelial function (Kotb et al. 2012). Furthermore, FMD tertile is an independent predictor of the presence of significant coronary artery stenosis in patients with poor glycemic control but not for those with good glycemic control (Chen et al. 2021). Impaired vascular reactivity is reversible with glucose-lowering agents and exercise (Shigiyama et al. 2017; Stojanović et al. 2016; Romualdi et al. 2008; Sugiyama et al. 2018; Baier et al. 2021; Dawson et al. 2013). The clinical implications are that interventions that lead to improved endothelial function as evidenced by aortic de-stiffening or improved vasoreactivity may be associated with reduced morbidity or mortality in T2DM.

The availability of multiple methods which address various aspects of endothelial physiology allows for a thorough approach for assessing endothelial function in T2DM. Although these techniques correlate with CV risk and some have been validated for CV risk stratification, their main disadvantage relates to the labor-intensiveness and need for standardized protocols for each analysis, preventing wide-spread clinical use.

Mini-dictionary of Terms

- **Augmentation index**. Ratio of augmentation pressure over pulse pressure, indicating proportion of central pulse pressure that is attributed to the arterial reflected wave. This reflects arterial stiffness as well as the diameter and elasticity of small arteries and arterioles.
- **Flow-mediated dilatation**. A noninvasive measure of endothelial function based on endothelium-dependent vasodilatation induced by increased sheer stress after a period of arterial occlusion in the forearm.
- **Laser Doppler flowmetry**. A noninvasive assessment of skin microvascular function, based on the change in wavelength of light when it is reflected by moving red blood cells.
- **Pulse wave velocity**. The speed of an arterial waveform as it transits across an arterial segment, indicating the degree of arterial stiffness and remodeling.
- **Reactive hyperemia**. The transient increase in blood flow that occurs following a brief period of ischemia induced by arterial occlusion. This reflects peripheral microvascular function.
- **Skin autofluorescence**. A noninvasive biomarker of cumulative skin-advanced glycation end products. It serves as an indicator of glycemic stress and correlates with diabetes-associated vascular complications.

Key Facts of Endothelial Dysfunction in Type 2 Diabetes Mellitus

- Endothelial dysfunction is a state of systemic endothelial activation characterized by impaired endothelium-dependent vasodilatation.
- This also represents a procoagulatory, proinflammatory, and proatherogenic profile that serves as a marker of increased cardiovascular risk.
- T2DM is associated with an increased risk of cardiovascular disease, which may be attributed to endothelial dysfunction as an early step in this pathogenic process.
- Hyperglycemia induces adverse effects on vascular biology, leading to oxidative stress, apoptosis, vascular injury, and impaired vasomotor responses.
- In patients with T2DM, halting or reversing endothelial dysfunction may be a critical target for preventing the onset of atherosclerosis and cardiovascular disease.

Summary Points

- Endothelial dysfunction, well-recognized to occur in T2DM, is characterized by a loss of endothelium-mediated vaso-relaxation in response to sheer stress and pharmacodynamic stimulation.
- Venous occlusion plethysmography is one of the earliest methods of assessing endothelium-dependent vasomotion but is limited by its semi-invasiveness and time-consuming setup.

- Flow-mediated dilatation is a widely utilized noninvasive method of assessing endothelial function, which has shown good accuracy and reproducibility, and correlates well with cardiovascular outcomes. However, standardized environmental conditions and subject preparation need to be adhered to.
- Peripheral artery tonometry is an alternative noninvasive measure of endothelial function also based on the concept of reactive hyperemia, often seen to be more simple and less technically challenging than FMD. However, FMD and PAT results are not interchangeable and may reflect the different circulations – for example, macrovascular versus microvascular endothelial function of conduit versus peripheral resistance arterioles, respectively.
- Aortic pulse wave velocity and augmentation index indicate a degree of arterial stiffness, which correlates inversely with endothelial function.
- Laser doppler flowmetry assesses a complex cutaneous microvascular response which may not be entirely endothelium-dependent. The technique is currently limited by a lack of standardization leading to low reproducibility.

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Measures of Endothelial Function in Type 2 Diabetes: A Focus on Circulatory Biomarkers

42

Caroline Wei Shan Hoong

Contents

Introduction 869
Circulatory Biomarkers as Measures of Endothelial Function 869
Nitric Oxide Metabolites and Asymmetric Dimethylarginine (ADMA) 869
High-Sensitivity C-Reactive Protein (hsCRP) 871
Adhesion Molecules and Chemokines 872
Endothelial Progenitor Cells (EPC) 873
Endothelial Microparticles (EMP) 874
Advanced Glycosylation End Products 875
Endocan 877
External Counterpulsation (ECP) Therapy on Measures of Endothelial Function 877
Applications to Diabetes 878
Mini-Dictionary of Terms 879
Summary Points 880
References 880

Abstract

Endothelial dysfunction occurs early in the development of atherosclerosis and predicts the development of cardiovascular events in T2DM. The identification of patients with preclinical atherosclerosis allows for risk stratification and targeted intervention for primary prevention. The use of circulating biomarkers of endothelial function is a promising alternative for clinical use, in comparison to vasomotor techniques described in the previous chapter, which are more cumbersome and operator-dependent. This chapter describes the measurement of circulatory biomarkers of endothelial function and their evidence for use in T2DM. Of these, high-sensitivity CRP is currently the most utilized and validated serum biomarker for cardiovascular risk stratification, while other circulating

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biomarkers such as asymmetric dimethylarginine, VCAM-1, ICAM-1, advanced glycosylation end products, circulating endothelial progenitor cells, endothelial microparticles, and endocan hold promise as novel biomarkers of different aspects of endothelial dysfunction in T2DM, provided their quantification can be standardized and validated. Lastly, we highlight the use of external counterpulsation as an example of a therapeutic modality that can be employed to modulate these measures of endothelial function.

Keywords

Endothelial dysfunction · Diabetes · Cardiovascular risk · Circulatory biomarkers · Nitric oxide · Asymmetric dimethylarginine · High-sensitivity CRP · Advanced glycosylation end products · Adhesion molecules · Endothelial progenitor cells · Endothelial microparticles · External counterpulsation

Abbreviations

ADMA	Asymmetric dimethylarginine
AGEs	Advanced glycation end products
Akt	Protein kinase B
AU	Arbitrary units
COX-2	Cyclooxygenase-2
ECP	External counterpulsation
ELISA	Enzyme-linked immunosorbent assay
EMP	Endothelial microparticles
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ESRF	End-stage renal failure
ET-1	Endothelin-1
FMD	Flow-mediated dilatation
FoxO1	Forkhead box O1
HbA1c	Glycated hemoglobin
HPLC	High-performance liquid chromatography
hsCRP	High-sensitivity C-reactive protein
ICAM-1	Intracellular adhesion molecule-1
iNOS	Inducible nitric oxide synthase
LC-MS	Liquid chromatography-mass spectrometry
MCP-1	Monocyte chemoattractant protein-1
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
PWV	Pulse wave velocity
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SAF	Skin autofluorescence
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor-α

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Introduction

Endothelial dysfunction predicts the development of cardiovascular events (Maruhashi et al. 2018). Its presence has also been observed patients without clinical atherosclerosis (Clarkson et al. 1997; Poredoš and Ježovnik 2015; Juonala et al. 2004) and correlates to the duration and progression of known cardiovascular risk factors (Clarkson et al. 1996; Hashimoto et al. 2021). As endothelial dysfunction occurs at an early stage in the atherosclerotic process, the identification of patients with preclinical disease allows for risk stratification and targeted intervention. Hence, the measurement of surrogate biomarkers of endothelial dysfunction have been of much research interest in recent years.

The previous chapter described the use of endothelial vasomotion, based on the principle of reactive hyperemia, as well as arterial stiffness and skin microvascular function as various representations of endothelium function. A broader functional assessment of the endothelium would encompass the study of endothelial-derived molecules which modulate other aspects of endothelial biology such as adhesion, inflammation, thrombosis, and repair. These circulating markers may complement the physiological vasomotor assessments of endothelial function. Furthermore, the simplicity of venous blood draw is an advantage over non-circulatory methods of assessment previously described which are far more labor-intensive. This chapter aims to describe the circulatory biomarkers as measures of endothelial function and discuss their clinical utility in type 2 diabetes mellitus (T2DM). Subsequently, we highlight external counterpulsation (ECP) as an example of a therapeutic modality that holds promise in the modulation of various non-circulatory as well as circulatory measures of endothelial function.

Circulatory Biomarkers as Measures of Endothelial Function

Nitric Oxide Metabolites and Asymmetric Dimethylarginine (ADMA)

Nitric oxide (NO) is a potent vasodilator derived from L-arginine and is one of the most important regulators of vascular tone. NO is produced by the activation of endothelial nitric oxide synthase (eNOS) by the effect of chemical agonists and shear stress on endothelial chemoreceptors and mechanoreceptors, respectively (Gutiérrez et al. 2013). Reduced NO bioavailability is one of the cardinal features of endothelial dysfunction, implicated in the upregulation of reactive oxygen species (ROS) and inflammatory cytokine production leading to the expression of adhesion molecules and activation of platelet adhesion and aggregation.

However, the measurement of NO is difficult because of its short half-life of a few seconds. It forms rapid chemical reactions with many biomolecules, for example,

nitrite and nitrate in the presence of oxygen (Csonka et al. 2015). Furthermore, circulating serum NO may not represent endothelial-derived NO in the vascular bed of interest, as eNOS-independent reduction of dietary or other endogenous sources of nitrates may also contribute (Csonka et al. 2015). Samples should be obtained in the fasted state, and careful preparation is required including blood deproteinization and reduction by the Griess reaction (Sun et al. 2003; Bryan and Grisham 2007; Metel'skaia and Gumanova 2005). Studies have quantified serum NO derivatives by high-performance liquid chromatography (HPLC), ultraviolet spectrophotometry or electrochemical methods (Möller et al. 2019), but results should be interpreted with caution, bearing in mind sampling conditions and preparation.

The NOS-NO pathway is complex – while eNOS (mainly expressed in endothelial cells and cardiomyocytes) constitutionally regulates vascular function in physiological states, transcription of inducible NO synthase (iNOS) is induced in pro-oxidative, pro-inflammatory states in cells ranging from endothelial cells, leucocytes, vascular smooth muscle cells, cardiomyocytes, and fibroblasts, which leads to pathological remodeling (Farah et al. 2018). In the absence of sufficient substrate L-arginine, cofactors and a suitable redox environment, NOS uncoupling occurs, leading to peroxynitrite and superoxide formation and oxidative stress-induced endothelial dysfunction (Varadharaj et al. 2015). In the clinical setting, serum NO metabolites have inconsistently been associated with cardiovascular outcomes (Bahadoran et al. 2019; Gumanova et al. 2017; Maas et al. 2017). Hence, the unlike in vitro quantification of NO in human endothelial cells stimulated from eNOS activity, prognostic interpretation of circulating serum NO metabolites on endothelial function and cardiovascular outcomes must take into account an iNOS-induced endogenous source of NO metabolites, accumulation in renal dysfunction, as well as exogenous exposure from diet and medication (Bahadoran et al. 2019).

Closely intertwined with the NO pathway is ADMA which acts as a circulating endogenous inhibitor of NO synthase by competing with L-arginine as the substrate, blocking the synthesis of NO for endothelial homeostasis. ADMA reduces endothelium-dependent vasodilatation and increases systemic vascular resistance (Achan et al. 2003). Its levels are inversely correlated with endothelial function as measured by flow-mediated dilatation (FMD) (Juonala et al. 2007). Elevated ADMA has been associated with carotid intima-media thickness, hypertension, peripheral artery disease, renal disease, and diabetic microvascular complications, and is postulated to lead to accelerated atherosclerosis (Bai et al. 2013; Yamagishi et al. 2008; Aldámiz-Echevarría and Andrade 2012; Gamil et al. 2020; Wilson et al. 2010).

In patients with T2DM, glucose-lowering therapy is associated with improvement in endothelium-dependent dilatation and a reduction in plasma ADMA levels (Yasuda et al. 2006; Asagami et al. 2002). Studies suggest that ADMA may be an important biomarker for the development of cardiovascular disease and all-cause mortality (Willeit et al. 2015; Zhou et al. 2017). As ADMA levels fall within a narrow range, good analytical precision is required to distinguish between normal and elevated (Tsikas 2008). HPLC is most often used but can be time consuming; enzyme-linked immunosorbent assay (ELISA) is faster but less reliable (Boelaert

et al. 2016; Martens-Lobenhoffer et al. 2005). Even though reference ranges are now available (Németh et al. 2017), standardization of techniques are required before ADMA can be reliably used as a biomarker for routine clinical practice (Tain and Hsu 2017).

High-Sensitivity C-Reactive Protein (hsCRP)

As a central orchestrator of endothelial dysfunction and atherosclerosis, vascular inflammation facilitates the interactions between cell adhesion molecules, chemotactic proteins, macrophages, modified lipoproteins that incite damage to the arterial wall, and progression of atherosclerotic plaques. hsCRP has emerged as validated measure of cardiovascular inflammation and an independent predictor of cardiovascular events both for primary and secondary prevention (Ridker et al. 2008; Carrero et al. 2019; Emerging Risk Factors Collaboration [ERFC] et al. 2010; ERFC 2012). hsCRP can be measured with an inexpensive high-sensitivity assay, is readily available, and has an established use in clinical guidelines (Arnett et al. 2019).

However, evidence is inconsistent, with some studies reporting only little incremental value for the prediction of cardiovascular events in addition to conventional risk factors (Danesh et al. 2004; Wilson et al. 2005). As CRP is an acute-phase reactant, elevated levels may not always be specific for cardiovascular disease. Furthermore, in patients with diabetes, its predictive value for CV disease is more controversial (Best et al. 2005; Kengne et al. 2012; Biasucci et al. 2009), deserving further study. Current lipid guidelines suggest the use of hsCRP as a cardiovascular risk enhancer, with levels >2.0 mg/l favoring the initiation of moderate-intensity statin in those with intermediate cardiovascular risk (Grundy et al. 2019). Some experts suggest a higher CRP threshold for predicting adverse outcome in T2DM (Bertoluci and Rocha 2017). In the EXAMINE trial in T2DM patients, baseline hsCRP >3.0 mg/dl predicted the development of major adverse cardiovascular events (Fig. 1, Hwang et al. 2018).

CRP is not only a predictor of CV risk but also a modulator of the endothelial dysfunction (Verma et al. 2003a). It inhibits eNOS activity while upregulating endothelin-1 (ET-1) (Hein et al. 2009), facilitates endothelial cell apoptosis and the release of plasminogen activator inhibitor-1, and limits endothelial progenitor cell survival and differentiation (Chen et al. 2008; Verma et al. 2004). T2DM has been widely accepted to represent a state of low-grade systemic inflammation (Ziegler 2005; Duncan et al. 2003). The proatherogenic effects of CRP in endothelial cells are exaggerated in the hyperglycemic state (Verma et al. 2003b). In subjects with metabolic syndrome, hsCRP is associated with an increased risk of developing T2DM, and its levels correlate with the degree of beta-cell dysfunction and insulin resistance (Pfützner and Forst 2006).

In addition, endothelial dysfunction plays an important role in the microvascular complications of diabetes. In the kidneys, glomerular endothelial cells, afferent and efferent arterioles are targets of vascular inflammation in diabetic nephropathy (Cheng and Harris 2014). hsCRP levels predicts incident diabetic nephropathy and

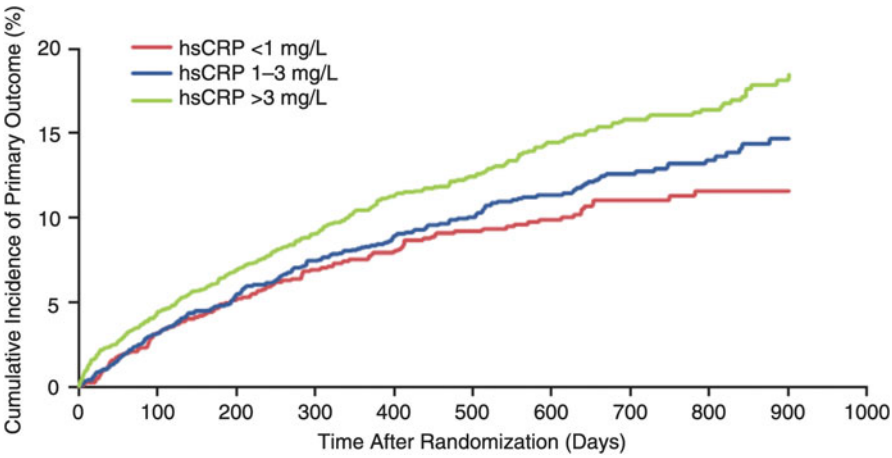

Fig. 1 T2DM patients with hsCRP > 3 mg/l have the highest risk of cardiovascular events. Time to the primary endpoint (major cardiovascular events) according to baseline hsCRP in the EXAMINE trial. From "High-sensitivity C-reactive protein, low-density lipoprotein cholesterol and cardiovascular outcomes in patients with type 2 diabetes in the EXAMINE (Examination of Cardiovascular Outcomes with Alogliptin versus Standard of Care) trial" by Hwang et al. (2018), licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/)

correlates with the degree of microalbumin secretion and severity of nephropathy, as well as other metabolic parameters of cardiovascular risk in T2DM (Sinha et al. 2019; Shaheer et al. 2017).

Adhesion Molecules and Chemokines

Intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin are adhesion molecules expressed by endothelial cells that are upregulated by cytokine activation. They facilitate the interaction between leucocytes and endothelial cells. Monocyte chemoattractant protein-1 (MCP-1) is a chemotactic cytokine produced by endothelial cells that mediate the activation of monocytes and T-cells and proliferation of vascular smooth muscle cells. These molecules contribute to vascular inflammation in the pathogenesis of atherosclerosis. Circulating levels of ICAM-1, VCAM-1, E-selectin, and MCP-1 can be measured in the serum and have been found to correlate with T2DM, metabolic syndrome, and development of cardiovascular disease (Chacón et al. 2007; Rubin et al. 2008; Hwang et al. 1997; Georgakis et al. 2019; Gregg et al. 2018; Rabkin et al. 2013; Song et al. 2007). Importantly, the predictive value of a raised MCP-1 for recurrent cardiovascular events is additionally prognostic over hsCRP levels (de Lemos et al. 2003; Blanco-Colio et al. 2021).

High glucose levels increase MCP-1 release, ICAM-1 and VCAM-1 expression, enhancing monocyte activation and interaction with the endothelium (Haubner et al.

2007; Luo et al. 2008; Ceriello et al. 1998). Hyperglycemia incites a series of metabolic activity within the cell that leads to the production of advanced glycation end products (AGEs) and ROS (Rhee and Kim 2018). AGEs disrupt glomerular homeostasis by inducing apoptosis of mesangial cells and stimulating MCP-1 and vascular endothelial growth factor (VEGF) secretion, leading to glomerular hyperfiltration (Yamagishi and Matsui 2010). The role of MCP-1 in progression of diabetic nephropathy has been demonstrated in human and animal studies (Tesch 2008; Murea et al. 2012; Tilak et al. 2010). Urinary MCP-1 independently predicts the risk of deterioration in renal function over urinary protein/creatinine ratio in patients with diabetes (Tam et al. 2009). In addition, adhesion molecules VCAM-1, ICAM-1, E-selectin, and chemokine MCP-1 are implicated in the pathogenesis of other diabetic microvascular complications such as diabetic retinopathy (Taghavi et al. 2019; Noda et al. 2012); however, these studies are largely preclinical studies and derived from vitreous samples. The evidence for serum MCP-1 as a biomarker for diabetic retinopathy and neuropathy is sparse currently but appears promising (Ozturk et al. 2009; Mussa et al. 2021)). Serum VCAM-1 and ICAM-1 are also emerging as potential biomarkers for predicting the development of microvascular and macrovascular complications in patients with T2DM (Bruno et al. 2008; Jude et al. 2002; Liu et al. 2015; Hegazy et al. 2020).

Endothelial Progenitor Cells (EPC)

Endothelial function reflects the homeostasis between endothelial damage and repair. EPCs are derived from the bone marrow. As they have the capacity to differentiate into mature endothelial cells, EPCs provide a circulating pool which can be easily mobilized at sites of endothelial damage, proliferate, and repair vascular endothelium. The number of circulating EPCs hence represents endogenous reserves to maintain and restore endothelial function, correlating well with vascular function as measured by flow-mediated dilatation (Hill et al. 2003). EPCs are quantified by flow cytometry based on surface expression of CD34 and CD133, and the endothelial marker VEGF-receptor 2 (Fadini et al. 2012). Accumulating evidence point towards circulating EPC number and function as biomarkers of cardiovascular risk, independent of traditional risk factors (Rigato et al. 2016; Werner et al. 2005). As an indicator of endothelial dysfunction, reduced and/or dysfunctional circulating EPCs are associated with peripheral artery disease, CAD, heart failure, and ischemic stroke (Lee and Poh 2014; Zhao et al. 2019), as well as increased cardiovascular risk in conditions associated with chronic inflammation such as systemic lupus erythematosus, rheumatoid arthritis, and T2DM (Menegazzo et al. 2012; Kahlenberg et al. 2011; Adawi et al. 2018; Fadini et al. 2017).

T2DM is associated with reduced numbers and functional impairment of circulating EPCs, demonstrating reduced mobilization from the bone marrow, diminished capacity for proliferation, adhesion to activated endothelial cells, and integration into vascular structures (Tepper et al. 2002; van Ark et al. 2012; Westerweel et al. 2013). In addition, circulating EPC levels negatively correlate with glycemic control and

degree of arterial stiffness (Yue and Lau 2011a). The reasons behind these observations are complex and multifactorial. A high glucose environment has been associated with triggering EPC apoptosis and enhancing senescence in vitro via the p38 mitogen-activated protein kinase and NO-dependent mechanisms (Chen et al. 2007; Kuki et al. 2006), while retardation of EPC growth and differentiation is modulated by the protein kinase B (Akt)/forkhead box O1 (FoxO1) pathway (Marchetti et al. 2006). AGEs in a hyperglycemic state can also impair the migration and adhesion of EPCs (Li et al. 2012). Downregulation of EPCs in T2DM was paralleled by a reduction in sirtuin-1 levels in early EPCs, being more pronounced in those with poor glycemic control (Balestrieri et al. 2013). Inhibition of sirtuin-1 signaling has been linked to the vascular remodeling, inflammation, and endothelial dysfunction (Man et al. 2019).

Mechanisms are yet to be fully elucidated; despite this, evidence is compelling for the involvement of EPCs in the pathogenesis of endothelial dysfunction in diabetes. In patients with T2DM, circulating EPC count predicts microvascular outcomes of nephropathy, retinopathy, and neuropathy (Rigato et al. 2015). Furthermore, it is associated with peripheral vascular complications and development of long-term cardiovascular outcomes in T2DM (Fadini et al. 2005, 2017). The use of some antidiabetic medications such as metformin, sitagliptin, pioglitazone, and canagliflozin have been associated with a restoration of EPC number and function (Ahmed et al. 2016; Yu et al. 2019; Esposito et al. 2011; Nandula et al. 2021). Besides its use as a prognostic indicator in conditions associated with endothelial dysfunction, recent interest has developed in its potential for therapeutic intervention (Pyšná et al. 2019; Kundu et al. 2021). The emergence of EPC-based therapies in particular for diabetes-associated ischemic complications such as acute myocardial infarction or critical limb ischemia are reviewed elsewhere (Dubský et al. 2017; Terriaca et al. 2021).

Endothelial Microparticles (EMP)

Microparticles are small submicron membrane vesicles released from platelets, leucocytes, and endothelium during cellular stress (VanWijk et al. 2003). They are quantified in the blood by flow cytometry. In a recent meta-analysis, patients with T2DM were found to have higher levels of EMPs than patients without diabetes (Li et al. 2016). Release of EMPs can be induced by inflammatory cytokines, ROS, thrombin, and plasminogen activator inhibitor (Leroyer et al. 2010). Activated endothelial cells release EMPs into the circulation, which further promotes the progression of endothelial dysfunction by their pro-inflammatory, procoagulant, and proapoptotic effects (Deng et al. 2016). Hence, elevated circulating EMP levels reflect endothelial injury and serve as a surrogate measure of vascular dysfunction.

EMPs are not only bystanders but also mediators of vascular dysfunction. EMPs inhibit the release of endothelial NO when obtained from patients with end-stage renal failure (ESRF), but not from healthy controls (Amabile et al. 2005). EMPs from patients with diabetes and vascular complications induce vascular hypoactivity,

via the induction of pro-inflammatory proteins, inducible NO-synthase, cyclooxygenase-2, and nuclear factor kappa B (NF-kB) activation (Tesse et al. 2005). Furthermore, EMPs increase in parallel with endothelial dysfunction in patients with diabetes correlate negatively with flow-mediated dilatation and may identify diabetes patients at risk of cardiovascular disease (Feng et al. 2010; Koga et al. 2005). This makes it a potential biomarker for vascular risk in T2DM.

However, its use in routine clinical practice is limited by a lack of standardization in measurement, including control of pre-analytical factors such as blood collection, sample transportation, washing steps, centrifugation, and storage temperature (Ayers et al. 2011; van Ierssel et al. 2010). Its quantification by flow cytometry is also variably influenced by the threshold for particle size detection, instrument settings, and the choice of specific antibodies (Robert et al. 2012; Duval et al. 2010). Developing standardized high-sensitivity flow cytometry protocols are important for ensuring reproducibility before they can be reliably used as clinical prognostic markers.

Advanced Glycosylation End Products

AGEs are a heterogenous group of macromolecules that increase in tissues with age from the nonenzymatic glycation of lipids and proteins, and its accumulation is accelerated in chronic diseases such as hypertension and diabetes (Ramasamy et al. 2011). In the vascular wall, AGEs modify collagen and elastin, upregulate inflammation in the extracellular matrix, leading to increased arterial stiffness (Senatus and Schmidt 2017).

In diabetes, high glucose levels result in glycation of plasma proteins and collagens, which alter their molecular conformation and interfere with their function. Increased AGEs have been linked to endothelial dysfunction in diabetes (Fig. 2) with resultant micro- and macrovascular complications (Singh et al. 2014). ACEs bind to their receptor RAGE on endothelial cells which leads to a cascade of signaling events that lead to enhanced oxidative stress, endothelial activation and expression of adhesion molecules, translocation of macrophages, and reduction of NO-dependent vasodilatation (Singh et al. 2014; Stirban et al. 2013).

Serum and plasma AGE levels have been strongly correlated with cardiovascular disease in clinical studies, particularly in patients with diabetes (Kiuchi et al. 2001; Saremi et al. 2017; Kilhovd et al. 1999). Measurement of AGEs in serum/plasma has been traditionally performed by HPLC or liquid chromatography-mass spectrometry (LC-MS) (Ashraf et al. 2015), but these are cumbersome for a large number of clinical samples. ELISA allows for more rapid detection. However, results are dependent on sample preparation, antibody affinity, and are limited by a lack of standardization and limited reproducibility (Ashraf et al. 2015; de Vos et al. 2016). Furthermore, their levels can be influenced by variable clearance in chronic kidney disease (Busch et al. 2010).

While AGEs can also be detected using immunohistochemical staining of a variety of tissues and cells, the need for invasive biopsies to obtain tissue samples makes it less convenient for use as a biomarker in clinical studies. Skin AGEs can be

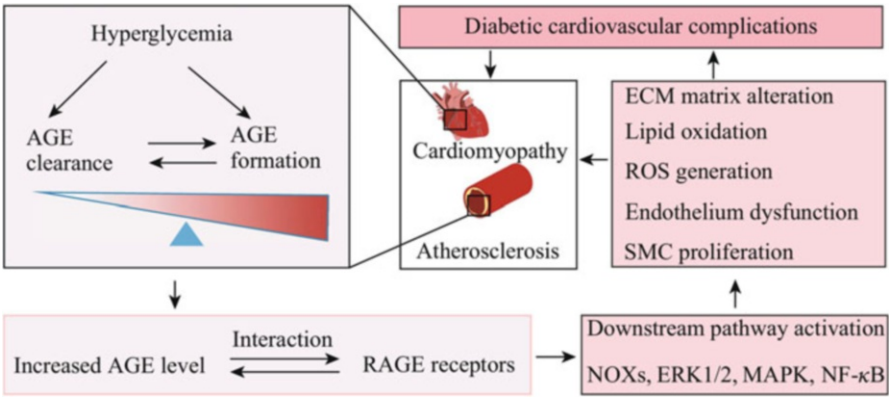

Fig. 2 The role of AGEs in cardiovascular complications in diabetes. Under hyperglycemic conditions, the balance between AGE formation and clearance is impaired, thereby resulting in increased AGEs. AGEs interact with AGE receptors to promote the development of diabetic cardiovascular complications such as endothelial dysfunction and atherosclerosis. From "Advanced Glycation End Products: Potential Mechanism and Therapeutic Target in Cardiovascular Complications under Diabetes" by Yang et al. (2019), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

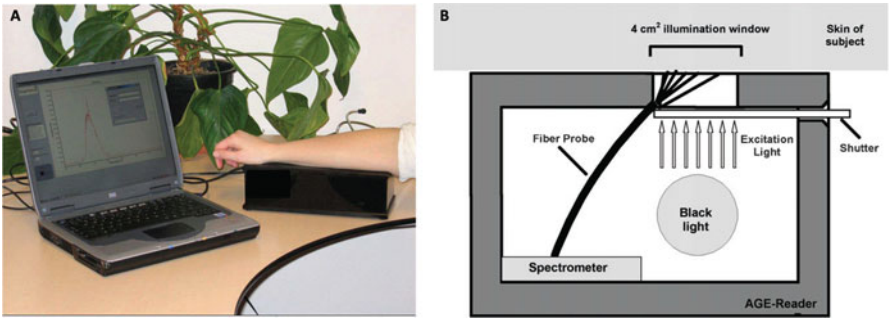

Fig. 3 Skin autofluorescence by AGE reader. (**a**) Autofluorescence reader in a clinical setting. (**b**) Schematic view of the autofluorescence reader. From "AGEs, autofluorescence and renal function" by Gerrits et al. (2009) with permission

measured noninvasively by SAF (skin autofluorescence) by the AGE Reader (DiagnOptics Technologies BV, Groningen, the Netherlands) as described by Meerwaldt et al. (2005). This is due to the autofluorescent properties of several AGEs. A skin surface area on the arm of approximately 4cm^2 is illuminated by multiple light-emitting diodes, and the autofluorescent light emitted is captured with a spectrometer and expressed in arbitrary units (AUs) (Fig. 3). Reference values exist for Caucasians and Asians (Lutgers et al. 2006; Yue et al. 2011b). SAF has been shown to be higher in patients with T2DM and is associated with the presence and severity of diabetes-associated vascular complications (Lutgers et al. 2006). As the half-life of skin collagen is 15 years, skin AGE levels may provide a longer-term

indicator of glycemic stress compared to glycated hemoglobin (HbA1c) (Verzijl et al. 2000). AGEs as measured by SAF have shown to correlate with vascular stiffening across all levels of glycemia (Birukov et al. 2021) and is additionally predictive of cardiovascular events in patients with T2DM (Lutgers et al. 2009). While measurement of AGEs by SAF remains as a promising biomarker for vascular dysfunction in diabetes, the majority of AGEs are not fluorescent and are thus not detected, and importantly, its use is limited in individuals with dark skin and the influence of creams and sunscreens, as well as conditions inducing severe vasoconstriction or vasodilatation (Ashraf et al. 2015; Noordzij et al. 2011).

Endocan

Endocan, or endothelial cell-specific molecule-1, is a soluble proteoglycan secreted by activated vascular endothelium, and regulates cell adhesion, differentiation, migration, and neovascularization (Kali and Shetty 2014). Many studies have demonstrated the use of plasma endocan levels as a novel biomarker of endothelial dysfunction and inflammation in multiple pathological states ranging from sepsis to T2DM, hypertension, cancer, autoimmune disease, chronic kidney disease, and cardiovascular disease, and correlated with inflammation severity and survival in these conditions (Pauly et al. 2016; Oktar et al. 2019; Kim et al. 2020; Balamir et al. 2018; Icli et al. 2016; Musialowska et al. 2018; Cox et al. 2015; Pawlak et al. 2015; Poon et al. 2019; Zhang et al. 2021). In T2DM, serum endocan levels are elevated in those with subclinical atherosclerosis as determined by carotid intima-media thickness, and positively correlates with HbA1c, suggesting the potential use of serum endocan as a biomarker for the early diagnosis of macrovascular injury in T2DM (Lv et al. 2017). ELISA kits are currently available for the measurement of serum endocan primarily in the research setting. As a relatively novel biomarker, more studies are needed to validate its use for the prognostication of micro- and macrovascular complications of T2DM.

External Counterpulsation (ECP) Therapy on Measures of Endothelial Function

ECP is described here as an illustrative example of a therapeutic modality which improves measures of endothelial function and how it translates to clinical outcomes. ECP is a noninvasive electrocardiogram-synchronized circulatory assist device for the treatment of ischemic cardiovascular disease. ECP is performed by sequential inflation of 3 cuffs on the lower extremities for improved hemodynamics and flow velocity which generates sheer stress with each compression/decompression cycle (Fig. 4). ECP therapy is typically delivered in 35 1-h sessions over 7 weeks.

ECP has been shown to induce the release of the vasodilator NO and reduce vasoconstrictors such as ET-1, with improvement in vascular reactivity (Shechter et al. 2003), and promote vasculogenesis (Bonetti et al. 2003). While some of the benefits for symptomatic angina relate to immediate hemodynamic effects, other

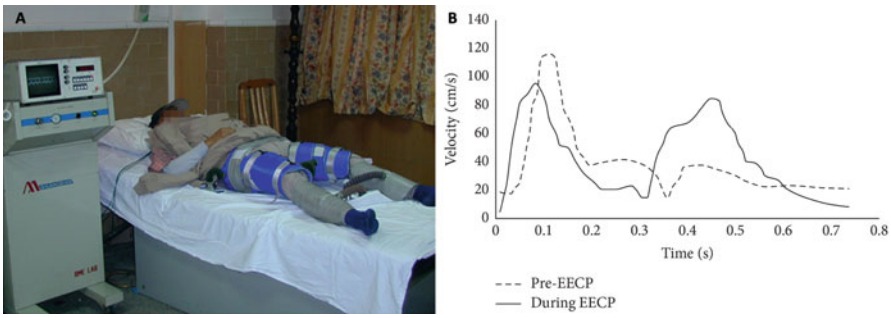

Fig. 4 Diastolic augmentation during ECP generates shear stress. (**a**) ECP treatment involves a set of cuffs wrapped around the lower extremities connected to an air compressor. (**b**) Blood velocity waveforms at the common carotid artery in a cardiac cycle before and during ECP, extracted from the images of ultrasound flow velocity spectrum. From "The Hemodynamic Effect of Enhanced External Counterpulsation Treatment on Atherosclerotic Plaque in the Carotid Artery: A Framework of Patient-Specific Computational Fluid Dynamics Analysis" by Du et al. (2020), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

benefits also derive from its ability to improve arterial stiffness and peripheral endothelial function, reducing oxygen demand (Braith et al. 2010). This is evidenced by improved pulse wave velocity (PWV), improved FMD, changes in endothelial-derived vasoactive agents such as an increase in NO, reduction in ET-1 and ADMA levels, as well as reduced circulating measures of inflammation such as hsCRP, MCP-1, TNF-α, and VCAM-1 (Braith et al. 2010; Casey et al. 2011). Furthermore, ECP therapy-induced improvement in FMD correlated with an increase in circulating EPCs and colony-forming capacity (Barsheshet et al. 2008; Liang et al. 2021), attenuating hypertensive vascular injury. These mechanisms likely underscore the ability of ECP to restore vascular endothelial integrity.

In T2DM, the benefits of ECP are less well established. A handful of studies report an improvement in fasting glucose, HbA1c, insulin resistance, and markers of oxidative stress and inflammation (Sardina et al. 2016a, b). However, another study in T2DM patients showed no improvement in reactive hyperemia (Hoong et al. 2020). In subjects with CAD undergoing ECP, the presence of diabetes was one of the factors associated with treatment failure (Erdling et al. 2008; Lawson et al. 2003). It is possible that only subgroups of patients with diabetes may benefit, such as those with longer duration of diabetes, greater degree of endothelial dysfunction, or higher insulin resistance at baseline (Hoong et al. 2020). More studies are required to clarify the use of ECP in patients with diabetes.

Applications to Diabetes

As a balance of vascular damage and repair occurs in the pathogenesis of endothelial dysfunction, circulating markers such as serum AGE, EMPs, and EPCs demonstrate the importance of vascular homeostasis in maintaining endothelial function. Besides

their role in predicting macrovascular complications, they may be additionally useful in predicting the development of microvascular complications in diabetes. In particular, measurement of circulating EPCs provide not only provide prognostic information on risk stratification in diabetes, but also appear to be promising in its role in as cell-based therapy for diabetes patients with critical limb ischemia (Beltrán-Camacho et al. 2021). This is especially important given high rates of amputation in patients with diabetic foot complications, leading to significant morbidity. These methods highlight endothelial dysfunction as an important target for the prevention and treatment of cardiovascular complications in diabetes (Lerman and Zeiher 2005).

The role of chronic inflammation in endothelial dysfunction and cardiovascular risk is highlighted by recent trials with the anti-inflammatory therapy canakinumab. Canakinumab is an interleukin-1ß inhibitor, which demonstrated large reductions in hsCRP and interleukin-6, paralleled by a lower incidence of recurrent cardiovascular events in patients with and without diabetes, despite little change in HbA1c (Ridker et al. 2017, 2012). Other circulating measures of chronic inflammation such as MCP-1, ICAM-1, and VCAM-1 also appear promising for cardiovascular risk stratification and target organ damage in diabetes.

Circulating biomarkers of endothelial function hold promise for the widespread clinical use for cardiovascular risk stratification and prognostication of microvascular complications in T2DM due to convenience and ease of measurement; however, adequate standardization and validation are required before they can be routinely applied and interpreted. Further studies are needed to identify populations that will benefit the most from these methods of risk stratification either individually or in combination, and to clarify whether any benefits from specific treatments are derived from an improvement in endothelial function.

Mini-Dictionary of Terms

- **Asymmetric dimethylarginine.** Analogue of L-arginine, a circulating endogenous inhibitor of nitric oxide synthase, hence blocking nitric oxide synthesis and impairing endothelial function.
- **Advanced glycosylation end products.** A group of proteins and lipids that result from glycation after exposure to sugars. Their accumulation in the vasculature is harmful and implicated in aging and in the pathogenesis of chronic diseases such as hypertension and diabetes.
- **Endothelial progenitor cells.** Circulating progenitor cells that have the capacity to differentiate into mature endothelial cells. They represent endogenous reserves to maintain and restore endothelial function.
- **Endothelial microparticles.** Small membrane vesicles released from platelets, leucocytes, and endothelium during cellular stress, reflecting degree of endothelial injury.
- **External counterpulsation.** A noninvasive circulatory device involving inflation of pumps on the lower extremities, generating sheer stress with each compression-decompression cycle and improving peripheral endothelial function.

Summary Points

- Endothelial dysfunction in T2DM represents a pro-atherogenic state that predisposes to heightened cardiovascular risk.
- Circulating biomarkers complement the physiological vasomotor assessments of endothelial function.
- Some of these biomarkers additionally provide prognostic information on the development of cardiovascular disease in T2DM, for example, ADMA, hsCRP, ICAM-1, VCAM-1, E-selectin, MCP-1, circulating EPCs, EMPs, AGEs, and endocan.
- hsCRP is a well-validated measure of cardiovascular risk. In T2DM, this represents chronic low-grade vascular inflammation exaggerated by the hyperglycemic state and higher thresholds may apply.
- Lower circulating EPCs and their functional impairment in T2DM reflect the degree of endothelial dysfunction in the hyperglycemic state. Circulating EPC count negatively correlates with glycemic control and predicts the development of micro- and macrovascular complications in T2DM.
- The convenience of a blood test offers the possibility of widespread adoption to guide clinical risk stratification over and beyond traditional cardiovascular risk factors; however, reference ranges and methodologies first require adequate standardization and validation before their routine use.
- External counterpulsation is a promising therapeutic modality that improves many measures of endothelial function, primarily in use for coronary artery disease. Its benefit in T2DM is currently investigational and deserves further study.

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Part V

Biomarkers in Specific Conditions or Scenarios of Diabetes

Biomarkers of Diabetes-Induced Nephropathy

43

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Contents

Introduction 893
Overview of Pathogenesis of Diabetic Nephropathy 893
Stages of Diabetic Nephropathy 894
First Stage 894
Second Stage 895
Third Stage 895
Fourth Stage 895
Fifth Stage 895
Conventional Biomarker of Diabetic Nephropathy 895
New Biomarkers of Diabetic Nephropathy 896
Biomarkers of Glomerular Damage 896
Biomarkers of Tubular Damage 898
Inflammatory Biomarkers 900
Biomarkers of Oxidative Stress Associated with Diabetic Nephropathy 901
Future Perspectives in the Diagnosis and Prognosis of Diabetic Nephropathy 903
NADPH Oxidase 903
MicroRNA (miRNAs) 903
Proteomics 903
Metabolomics 904
Genomics 904

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Mini-Dictionary of Terms 904
Key Facts of Biomarkers of Diabetes-Induced Nephropathy 905
Summary Points 905
References 905

Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease. Therefore, the assessment of renal function and early diagnosis of glomerular and tubular injuries is very important measure in the management of type 1 and type 2 diabetic patients. The diagnosis of DN for years has been routinely determined by the presence of microalbuminuria (MA). Several studies have showed that presence of MA may be transient and does not necessarily reflect permanent kidney damage. There could also be glomerulo-tubular damage in diabetic patient without presenting with albuminuria. Decline in the renal function, cellular and extracellular derangements in both the glomerulus and tubules has been associated with array of biological markers which could be of help in the diagnosis, prognosis, and the overall management of the affected patients. Identifying more biomarkers of both research and clinical importance to diagnose and predict progression of kidney damage in diabetics is necessary.

Keywords

Diabetes · Diabetic kidney disease · Diabetic nephropathy · Biomarkers

Abbreviations

AGE	Advanced glycated end-products
AUC	Area under curve
DAG	Diacyl glycerol
DKD	Diabetic kidney disease
DM	Diabetic mellitus
DN	Diabetic nephropathy
ECM	Extracellular matrix
GFR	Glomerular filtration rate
IgG	Immunoglobulin G
L-PGDS	Lipocalin-type prostaglandin D synthase
MA	Microalbuminuria
MAPK	Mitogen-activated protein kinase
NADPH	Nicotinamide adenine di-nucleotide phosphate hydrogen
NF-κb	Nuclear factor kappa b
PKC	Protein kinase C
RAGE	Receptors for advanced glycated end-products
ROS	Reactive oxygen species
TGF-β1	Tumor growth factor beta 1
VEGF	Vascular endothelial growth factor

Introduction

Diabetes mellitus (DM) is an oxidative and metabolic disorder of carbohydrate primarily, but metabolism of protein and lipids are affected in complicated cases (Ajilore et al. 2021). It is characterized by increase in blood sugar above normal level due to absolute or relative insulin deficiency. The primary factor in the development of diabetic complications is persistent chronic hyperglycemia (Aronson and Rayfield 2002). DM is associated with diseases of the eyes, kidney, heart, and liver. Some of the associated biochemical complications are hypoglycemia, hyperglycemic crisis, diabetic ketoacidosis, and hyperglycemic hyperosmolar state. Diabetic kidney disease (nephropathy) is a leading cause of chronic renal failure with increase in both morbidity and mortality. It is characterized by decreased glomerular filtration rate (GFR), increased glomerular basement membrane thickness, collapsed glomerulus, tubulo-interstitial fibrosis, decreased excretion of albumin, and decreased creatinine clearance (Bonventre 2012; Letizia et al. 2017; Ajilore and Adesokan 2018). Persistent and uncontrolled hyperglycemia generates oxidative stress responsible for the development of pathological features of diabetic disorder of the kidney (Ajilore and Adesokan 2018). Recent researches have provided new insights into the dynamics of diagnostic and prognostic biomarkers of diabetic nephropathy. This chapter focuses on the different biomarkers being used in the assessment of various stages of diabetic kidney disease.

Overview of Pathogenesis of Diabetic Nephropathy

Hyperglycemia-induced tissue damage (Alter et al. 2012) is the main insult responsible for pathological changes in the nephron of diabetic patients (Fang et al. 2012). There is oxidative stress when there is imbalance between prooxidant and antioxidant equilibrium (intracellular antioxidants and antioxidant enzymes) (Palacios 2011). Persistent hyperglycemia causes superoxide overproduction in the electron transport chain in the mitochondria which serves as the main mechanism for the development of diabetes at the molecular level. There is also evidence of increase in NADPH oxidase activity which is responsible for ROS production in the diabetic kidney disease (Jomova et al. 2011).

Activation of protein kinase C (PKC) pathway results as well in the production of ROS in diabetes. In addition, ROS has been reported to activate PKC, MAPK, JAK/STAT, and transcription factors (NF-κb, AP-1, and SP-1) and upregulate TGF-β1 and fibronectin levels leading to extracellular matrix (ECM) accumulation seen in diabetic disease of the kidney. NADPH oxidase activity is also implicated in the development of diabetic disease of the kidney. PKC has also been demonstrated to be involved in the activation of NADPH oxidase by high blood glucose and free fatty (Jomova et al. 2011). PKC inhibitors effectively inhibit hyperglycemia and free fatty acid-induced ROS generation. The signaling pathways being regulated by ROS

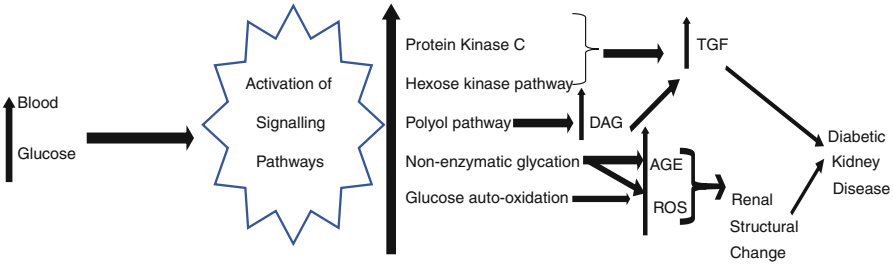

Fig. 1 Pathogenesis of diabetic kidney disease. Hyperglycemia-induced tissue damage via activation of signaling pathways. *TGF* tumor growth factor, *DAG* diacyl glycerol, *AGE* advanced glycated end-products, *ROS* reactive oxygen species

are suggested to be responsible for deposition of ECM in diabetic kidney disease. Hyperglycemia generates ROS which activate signal transduction cascades (PKC, MAPK, and JAK/STAT) and transcription factors (NF-kB, AP-1, and Sp1) and upregulates TGF-β1 and fibronectin in renal cells. Besides its function in upregulating synthesis of ECM, ROS are also involved in the degradation of ECM and also in transition of epithelium and mesenchyme in the tubular epithelial cells which leads the widening of glomerular space and that of tubule-interstitial expansion (Bai et al. 2012). It has been shown that ROS that are dichlorofluorescein sensitive increases in the glomerulus in streptozotocin-induced diabetic rats (Wang et al. 2012). Another pathophysiology for the development and progression of diabetic disease of the kidney is through advanced glycated end-products (AGE). AGE produce ROS directly or through its receptor, and ROS also promote formation of AGE (Lai et al. 2012). When receptor for AGE (RAGE) is overexpressed, development of diabetic kidney and eye diseases are exaggerated. RAGE can also be inhibited by formation of AGE formation. Intracellular antioxidants can also inhibit upregulation of TGF-β and fibronectin induced by hyperglycemia (Lin et al. 2018) and thereby reduce the resulting oxidative stress by increasing activities of antioxidant enzymes (Fig. 1).

Stages of Diabetic Nephropathy

Disease of the kidney in DM affects about 25–40% of type 1. There are five stages of diabetic kidney disease based on the evaluation of the renal function to explain the progression of the disease.

First Stage

There is hyperfiltration from increased demand on the neurons. This leads to increase in rate of filtration of the glomerulus (GFR) and glomerular hypertrophy.

Second Stage

In the second stage, GFR remains elevated or normal, but the assault to the glomerulus has progressed. There is significant microalbuminuria due to persistent hyperfiltration. This is evidenced by expansion of the mesangium or thickening of the basement membrane. Patients in the second stage excrete >30 mg urine albumin per day. If microalbuminuria persists, chronic disease of the kidney is developed. It is advisable that all DM patients should go for routine microalbuminuria yearly.

Third Stage

In stage 3, microallbuminuria persists and the damage to the glomerulus has progressed to clinical albuminuria. The patient urine is tested positive using dipstick at this stage. The urine contains >300 mg of albumin per day. Development of hypertension usually occurs during the third stage. There is also sclerosis of the mesangium.

Fourth Stage

This occurs in the late stage of DM. There is overt-proteinuria and hypertension. There is also progressive sclerosis of the glomerulus with increasing micro-albuminuria. The kidney begins to fail since capability to filter has started to decline, and there is also increase in urea nitrogen and creatinine in blood. Ten percent of GFR is lost every year.

Fifth Stage

This is the chronic stage of renal disease. Glomerular fitration rate, GFR, is <10 mL/min). There is glomerular fibrosis and sclerosis. Renal replacement therapy (i.e., hemodialysis, dialysis of the peritoneum, renal transplantation) is the mainstay of treatment (Romesh 2000).

Conventional Biomarker of Diabetic Nephropathy

Microalbuminuria is the earliest standard routine biomarker to detect diabetic nephropathy (DN) in the early stage. Its accuracy is a subject of concerns because not all diabetics with microalbuminuria will end up with end-stage renal disease (Adler et al. 2003). There could be renal dysfunction before the appearance of microalbuminuria in few cases, but the presence of microalbuminuria suggests ongoing kidney damage (Uwaezuoke 2017).

New Biomarkers of Diabetic Nephropathy

Biomarkers of DN are classified based on the source and pathological changes involved into the following:

A. Glomerular damage biomarkers
B. Tubular damage biomarkers
C. Inflammatory biomarkers
D. Oxidative stress biomarkers

It is of note that the classification above is dynamic. There is an overlap in the classification. An inflammatory biomarker could be as well listed under biomarkers for glomerular damage, etc. The sensitivity and specificity of any biomarker can be assessed according to its diagnostic accuracy using AUC (area under curve) criteria.

Biomarkers of Glomerular Damage

The following are markers of damage to the glomerulus: transferrin, IgG, immunoglobulin G ceruloplasmin, type IV collagen, laminin, glycosaminoglycans (GAGs), lipocalin-type prostaglandin D synthase (L-PGDS), fibronectin, podocytes-podocalyxin, and VEGF (Table 1). Appearance of transferrin in the urine during DN progression is positively associated with albumin-creatinine ratio (ACR) but negatively correlated to glomerular filtration rate (GFR) (Al-Rubeaan et al. 2017). Transferrin is a protein with molecular weight of 76.5 kDa. This is responsible for its low ionic load which allows it to freely pass through the glomerular membrane (Narita et al. 2004; Ito et al. 2008). Urinary transferrin has been demonstrated as a more reliable glomerular biomarker than albuminuria.

Immunoglobulin G (IgG) is an anionic protein in the serum with molecular weight of 150 kDa which is synthesized secondary to immune response. Its high molecular weight makes it difficult to pass the glomerular barrier (Gohda et al. 2012). IgG can be excreted in urine of non-albuminuric type 2 diabetes (DM) patient simultaneously with high levels of urinary transferrin and ceruloplasmin (Narita et al. 2004). Ceruloplasmin is a negatively charged copper-carrying protein with relatively high molecular weight (HMW) of 151 kDa. Its high molecular weight and its being negatively charged make it difficult to pass the glomerulus. It is secreted in the urine simultaneously with increased transferrin IgG in non-albuminuric type 2 DM patient (Narita et al. 2004). High level of ceruloplasmin in urine has positive correlation albumin excretion and predicts the development of microalbuminuria in previously non-albuminuric type 2 diabetes patients.

Type IV collagen is a constituent of glomerular basement membrane and mesangial matrix. It is also seen in proximal tubular cells and podocytes (Adler et al. 2000). Its elevated level is seen in normoalbuminuric type 1 DM patients. Type IV collagen synthesis is stimulated by persistent hyperglycemia and its excretion may be an early indicator diabetic renal injury (Fiseha 2015). Type IV collagen in the

Table 1 Glomerular biomarkers associated with diabetic nephropathy

Biomarker	T1DM	T2DM	Pre micro-albuminuria	Method of Detection	Clinical Significance
Transferrin	+	+	+	Urine	Predictor of early stage; increased before development of microalbuminuria
Immunoglobulin G	+	+	+	Urine	Associated with the progression of glomerular diffuse lesions
Ceruloplasmin	+	+	+	Urine	Suggest renal hemodynamic changes, such as increased interglomerular hydraulic pressure
Type IV collagen	+	+	+	Urine	Predictor of advanced stage of DN; associated with a faster decline in eGFR
Laminin	+	+	+	Urine	Has association with NAG (N-acetyl-beta-D-glucosaminidase) and alpha-1-microglobulin
Glycosaminoglycans	+	+	+	Urine	Associated with other tubular markers
Lipocalin-type prostaglandin D synthase		+	+	Urine	It predicts renal lesions, but less relevant as an early biomarker of DN
Fibronectin	+	+	–	Urine	
Podocytes-podocalyxin, and VEGF	+	+	–	Serum/ urine	Predictor of progression; increased during the earlier stage of DN and shown to significantly correlate with urinary albumin excretion

T1DM type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus
+ = Presence, – = Absence

urine could also be an indicator renal damage in type 2 DM patient (Okonogi et al. 2001). High level type IV collagen excretion in urine has also been reported in pre-diabetic normoalbuminuric patients (Takizawa et al. 1998). Urinary type IV collagen has also been found to show more sensitivity than albuminuria (Kotajima et al. 2000).

Laminin is an HMW glycoprotein with 400–900 kDa. It is a constituent of the glomerular basement membrane and mesangium. Increased excretion of laminin in urine of normoalbuminuric type 2 DM patients is positively associated with N-acetyl-beta-D-glucosaminidase, alpha-1-microglobulin, and type IV collagen excretion (Banu et al. 1995; Papadopoulou-Marketou et al. 2017). Glycosaminoglycans (GAGs) are low molecular weight (LMW) (13 and 30 kDa) constituents of glomerular basement membrane, tubular basement membrane, and extracellular matrix. Their excretions in the urine are also seen in normoalbuminuric DM patients. High level of GAGs in the urine is associated with excretion of tubular markers like Tamm-Horsfall protein, which expresses a distal tubular dysfunction as well (Ueta et al. 1995; Torffvit 1999). L-PGDS is an LMW (20–31 kDa) secretory protein of the lipocalin family which is implicated in the metabolism of arachidonic acid and synthesis of prostaglandins. It is a glomerular biomarker which reflects increased permeability. High urine level of L-PGDS is seen in normoalbuminuric type 2 DM patients and it is a very useful biomarker to predict future development of albuminuria (Cohen-Bucay and Viswanathan 2012; Davani et al. 2015).

Fibronectin, an HMW protein with 440 kDa, is a constituent of glomerular extracellular matrix. High urinary level has been implicated in macroalbuminuric types 1 and 2 DM patients. It is associated with progressive diffused glomerular lesions like glomerulosclerosis and fibrosis (Takahashi 1995; Fagerudd et al. 1997; Cao et al. 2016). Nephrin is a transmembrane protein found in the slit diaphragm of the glomerular filtration barrier while podocalyxin, a transmembrane protein member of the CD34 family is the main component of the glycocalyx of podocytes in the glomerulus (Lever and Sheer 2010; Hara et al. 2012). Wang et al. (2007) have described the presence of nephrin in the urine of type 2 DM patients with proteinuria and reduced GFR. Presence of nephrin in urine (nephrinuria) is as a result of slit diaphragm injury related to injury of the podocytes and is considered as a biomarker of early injury of the glomerulus in types 1 and 2 diabetic patients. In DN, nephrinuria could be caused by nephrin dysregulation in DM patients even before appearance of microalbuminuria (Patari et al. 2003; Jim et al. 2012). VEGF (Vascular endothelial growth factor) is also a podocyte biomarker that is affected in podocyte impairment in DM (Petrica et al. 2014).

Biomarkers of Tubular Damage

Microalbuminuria which is the gold standard biomarker for glomerular injury in DN has also been considered as biomarker of tubular injury in DN. The tubular biomarkers discussed under this section have proved that tubular injury can be present early in DN, even before glomerular injury. These biomarkers include neutrophil gelatinase-associated lipocalin (NGAL), α-1-microglobulin, kidney injury molecule 1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), cystatin C, and liver-type fatty acid-binding protein (L-FABP) (Table 2).

Neutrophil gelatinase-associated lipocalin NGAL is an LMW protein (25 kDa) that belongs to the lipocalin protein family which is mainly produced in the renal

Table 2 Tubular biomarkers associated with diabetic nephropathy

Biomarker	T1DM	T2DM	Pre micro-albuminuria	Method of Detection	Clinical Significance
Neutrophil gelatinase-associated lipocalin	+	+	+	Serum/ urine	High level reflects structural damage of renal cells
α-1-microglobulin	–	+	+	Urine	It predicts early tubular injury in normoalbuminuric diabetic patient
Kidney injury molecule-1	–	+	+	Urine	It predicts early tubular injury in normoalbuminuric diabetic patient
N-acetyl-β-D-glucosaminidase (NAG)	+	+	+	Serum/ urine	Predictor of early stage DN; associated with normoalbuminuric and micro-albuminuric stages; increased in parallel with the severity of disease
Cystatin C	–	+	+	Urine/ serum	It is useful for early detection of the decrease in glomerular filtration rate
Liver-type fatty acid-binding protein (L-FABP)	+	+	+	Urine	The values of urinary L-FABP increase with the decline of renal function. It is an independent predictor of the progression of DN

T1DM type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus
+ = Presence, – = Absence

tubules when there is structural kidney injury (but a lesser amount is also produced in the lung, trachea, stomach, and colon) and excreted in urine (Devarajan 2010; Thrailkill et al. 2010; Nauta et al. 2011). Serum NGAL is found in early tubulo-interstitial damage even in non-albuminuric diabetic patients (Lacquaniti et al. 2013; Papadopoulou-Marketou et al. 2014). Urinary NGAL can be used to differentiate intrinsic acute kidney injury (AKI) from pre-renal acute injury. In AKI, urinary NGAL concentration is increased before increased in serum creatinine concentration (Mishra et al. 2004). Urinary NGAL is a marker of structural damage and not of renal function since increase in its level could occur in the absence of deficit in the renal function (Papadopoulou-Marketou et al. 2017). NGAL can also be produced to a lesser extent in other tissues like heart but it is excreted in the urine (Devarajan 2010). Therefore, high level of this biomarker in the serum could be a prognosis of cardiovascular co-morbidity (Papadopoulou-Marketou et al. 2017).

Alpha-1-microglobulin is an LMW serum protein (27-kDa). This favor it being filtered easily through the glomerulus but reabsorbed in the proximal tubule. Tubular dysfunction in DN leads to alteration in its reabsorption with increased excretion in the urine (Kubisz et al. 2015; Papadopoulou-Marketou et al. 2017). Alpha-1-microglobulin is cheap and effective biomarker to predict DN in early stages of DM (Shore et al. 2010). Kidney injury molecule-1 (KIM-1) is a membrane protein found in the proximal convoluted tubular cells. This membrane glycoprotein is a sensitive biomarker for acute kidney injury and its increased level in urine has been observed in normoalbuminuric type 2 DM patient (Bonventre 2014). Urinary level of KIM-1 increases as albuminuria increases (Uwaezuoke 2017).

N-acetyl-β-D-glucosaminidase (NAG) is a proximal tubular cell lysosomal enzyme (Gluhovschi et al. 2016). NAG is a reliable prognostic marker of diabetic kidney injury. Its HMW impairs its being freely passed through the glomerular filtration barrier, hence its increased level in urine (Ajilore et al. 2021). NAG is excreted in urine throughout the course of DM in both type 1 and type 2 individuals, and even before the appearance of microalbuminuria (Kim et al. 2016). Cystatin C is a protein of LMW (13 kDa) which is produced by the body's nucleated cells (Jeon et al. 2011). Its LMW makes it to be easily filtered by the glomerulus but it is reabsorbed by the proximal tubular cells where it is metabolized. Cystatin C serum level is neither dependent on the muscle mass nor the diet as it is observed in case creatinine (Agnieszka et al. 2015). Serum cystatin is a sensitive biomarker in detection of mild glomerular injury while urinary cystatin C indicates tubular injury. It increases early in diabetes and prediabetic nephropathy (Gluhovschi et al. 2016). Liver-type fatty acid-binding protein (L-FABP) is a LMW (14 kDa) protein involved in the metabolism of long-chain fatty acids (Papadopoulou-Marketou et al. 2014). It is expressed primarily in the liver but can be expressed secondarily in renal proximal tubular cells in case of tubular injury. The urinary excretion of this protein is elevated in patients even before the development of glomerular damage or albuminuria (Campion et al. 2017).

Inflammatory Biomarkers

In DN, inflammation of the kidney results in the release of inflammatory cells (interleukins and cytokines) such as TNF-α, MCP-1, TGF-β1, IL-1β, IL-6, and IL-8 that aggravates the tissue injury (Table 3). TNF-α, an important cytokine, is produced by macrophages, renal tubular cells, and glomerular mesangial cells during hyperglycemia. TNF-α causes accumulation of extracellular matrix in glomerulus and tubules leading to alteration of glomerular filtration, tubular permeability, and reabsorption (Campion et al. 2017). Clinical studies have shown that serum and urinary concentrations of TNF-α in diabetic patients with renal dysfunction increases with progression of the disease. Serum and urinary TNF-α could predict the progression of DN to ESRD independent of albuminuria status of the diabetic patient (Gohda et al. 2012; Niewczas et al. 2012).

Table 3 Inflammatory biomarkers associated with diabetic nephropathy

Biomarker	T1DM	T2DM	Pre micro-albuminuria	Method of Detection	Clinical Significance
Tumor Necrosis Factor Alpha (TNF-α)	+	+	+	Serum/ urine	Predictor of DN progression to ESRD and GFR loss; associated with the presence and severity of microalbuminuric stage
Monocyte Chemoattractant Protein-1 (MCP-1)	+	+	+	Urine	Predictor of progressive renal disease; correlated significantly with albuminuria levels; accelerate nephropathy by increasing inflammation and fibrosis; potential for therapeutic target for treating DN
Tumor Growth Factor Beta TGF-β1	+	+	−	Serum	Predictor of DN progression to ESRD; associated with macroalbuminuria and risk of mortality
Interleukins (IL-1β, IL-6, IL-8, IL-18)	+	+	−	Serum/ urine	Predictor of DN progression; strongly associated with future risk of early progressive renal decline

T1DM type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus
+ = Presence, − = Absence

The infiltration of inflammatory cells like monocytes and macrophages in DM is the hallmark of the progression to DN. Monocyte Chemoattractant Protein-1 (MCP-1) is a cytokine secreted by mononuclear leukocytes, cortical tubular epithelial cells, and podocytes during inflammation of the kidney, glomerulo-tubular damage, fibrosis, and atrophy (Wada et al. 2000).

Biomarkers of Oxidative Stress Associated with Diabetic Nephropathy

Oxidative stress is key to the development and progression of kidney disease in diabetes mellitus (Ha and Lee 2001; Gluhovschi et al. 2016). Few biomarkers found in this class are 8-oxo-7,8-dihydro-2-deoxyguanosine (8oHdG), haptoglobin (Hp), pentosidine, uric acid, and redox-regulating protein, p66Shc (Table 4). 8-oxo-7,8-dihydro-2-deoxyguanosine (8oHdG) is found in the urine of diabetic patient. The production of 8oHdG is as a result of oxidative damage to DNA, and the marker is excreted in the urine unmetabolized. Urinary 8oHdG is a good clinical predictor of

Table 4 Biomarkers of oxidative stress associated with diabetic nephropathy

Biomarker	T1DM	T2DM	Pre micro-albuminuria	Method of Detection	Clinical Significance
8-oxo-7,8-dihydro-2-deoxyguanosine (8oHdG)	+	–	+	Urine	Useful clinical marker to predict the development of DN, and its high urinary excretion is significant of DN progression
Haptoglobin (Hp)	+	–	+	Urine	Predictor of DN progression to ESRD and reduced GFR
Redox-regulating protein p66Shc	+	+	+	Plasma/ renal biopsy	Predictors of the degree of tubular damage. Therapeutic target in DN
Pentosidine	–	+	+	Serum/ urine	Predictor of progression; influenced, by glycemic levels and renal function; Biomarker of microvascular complications and diabetic cardiovascular risk
Uric acid	+	+	+	Serum	Predictor of progression; Associated with various stages of DN, onset and progression; Potential target for therapeutic intervention in diabetes

T1DM type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus
+ = Presence, – = Absence

both development and progression of DN in diabetic patients (Hinokio et al. 2002; Wu et al. 2004).

Haptoglobin (Hp), an alpha-2 sialo-glycoprotein, is another marker of oxidative stress that can be used to assess the development of DN in diabetic patient. Oxidative stress resulted from continuous hyperglycemia in diabetes generates free oxy-Hb and met-Hb causing increase in the level of this marker since Hp functions as an antioxidant and tends to bind Hb (Ajilore et al. 2021) to prevent iron loss through the kidney. Urinary concentrations of Hp rise following a drop in GFR before the development of macroalbuminuria. The predictive ability of urinary Hp appears to be equal to that of the albumin excretion rate (Papadopoulou-Marketou et al. 2017).

The formation of advanced glycated end products (AGEs) in the cell is due to oxidative stress generated as a result of persistent hyperglycemia. The rate of accumulation of these AGEs like N(6)-carboxymethyllysine and pentosidine is increased in diabetes and as well correlate with the severity of complications in diabetic patients (Campion et al. 2017). Pentosidine is one of the best chemically characterized AGE compounds, and there is elevated urine and serum concentrations in type 2 DN patients with microalbuminuria and renal dysfunction (Piarulli et al. 2009).

Uric acid (UA), a molecule involved in oxidative stress, also plays a role in the progression of DN. UA is the final enzymatic product in the degradation of purine nucleosides.

The redox-regulating protein, p66Shc, is an adaptor protein localized within the mitochondrial fraction. It functions in reducing equivalents of the mitochondrial electron transfer chain by the oxidation of cytochrome C. p66Shc is also involved in mitochondrial reactive oxygen species generation and apoptosis. Increased expression of p66Shc is associated with development of DN (Bock et al. 2013; Xu et al. 2016).

Future Perspectives in the Diagnosis and Prognosis of Diabetic Nephropathy

NADPH Oxidase

Hyperglycemia and other disease conditions like dyslipidemia and hypertension create a diabetic environment that generates ROS, e.g., superoxide, and activates signaling pathways (Fig. 1) resulting in oxidative stress and causing damage to both endothelial and epithelial cells of renal tubules. NADPH oxidase plays a key role in the oxidative stress. One of the isoforms of this enzyme, NOX-4, is key to the mechanism of ROS generation (Gorin and Block 2013). Associations between NOX-4 and DN need to be investigated since deletion of NOX-4 gene could abate progression of diabetic kidney disease.

MicroRNA (miRNAs)

miRNAs have been one of the latest novel diagnostic biomarkers and drug targets in the pathogenesis of many diseases including DN. They are small noncoding RNA (20–30 nucleotides) which inhibits protein translation, induces degradation of mRNAs, and thereby acts as regulators of gene expression (Simpson et al. 2016). They have been investigated to be involved in the post-transcriptional regulation of many gene expressions and control of many biological processes such as apoptosis, DNA repair, oxidative stress response, cancer, and cellular development (Campion et al. 2017). miRNAs that target genes associated with inflammation, fibrosis, and oxidative stress seem to be promising new tools in the diagnosis, prognosis, and progression of DN (Xu et al. 2014; Eissa et al. 2016; Barutta et al. 2017).

Proteomics

Proteomics is another promising diagnostic and prognostic tools or methods. Proteomics involves use of large-scale experimental analysis of proteins, through protein purification and mass spectrometry (Papadopoulou-Marketou et al. 2017). The

method introduces several biomarkers of AKI and CKD, found in urine, blood, or tissues. Proteomics is a noninvasive tool that could be used in a preclinical phase of kidney diseases. A study revealed collagen fragments as a promising biomarker which can be used to detect DN 3–5 years before the onset of microalbuminuria (MA) in diabetic patients (Zurbig et al. 2012). Fetuin-A is another biomarker responsible for the development of MA and reduced GFR in DN (Inoue et al. 2013).

Metabolomics

Findings of metabolomic profiling in some diabetic patients have revealed the importance of the method regarding progression of DN to ESRD. A study showed that the levels of some metabolites like 4-oxopentanoate, glucoronate, 2-hydroxyisobutyrate, 5-oxoproline, pimelate, N-acetylneuraminate, 3-methylhistidine, phthalate, trp, hippurate, and 3-hydroxy-3-methylglutarate have good predictive values regarding the progression of diabetic kidney disease, DKD (Kimura et al. 2012). In another study where a metabolomics-based approach was used in the identification of potential biomarkers of DKD, the main metabolites found were products of lipid metabolism like esterified and non-esterified fatty acids, carnitines, phospholipids, branch-chain amino acid and aromatic amino acid metabolism, carnitine and tryptophan metabolism, nucleotide metabolism (purine, pyrimidine), the tricarboxylic acid cycle, or uremic solutes (Colhoun and Marcovecchio 2018).

Genomics

Genome-wide association studies (GWAS) for single-nucleotide variations (SNVs) have confirmed genetic predisposition in relatives of patients with DKD (Papadopoulou-Marketou et al. 2017). Chromosome 18 has been implicated as the site of SNVs responsible for DKD. Also, an association between the carnosinase D18S880 microsatellite polymorphism and DKD susceptibility has been confirmed in Caucasians (Conserva et al. 2016).

Mini-Dictionary of Terms

- ***Dichlorofluorescein.*** *It is an organic dye of the fluorescein family, being substituted at the 2 and 7 positions by chloride. Fluorescein is an organic compound and dye which is widely used as a fluorescent tracer.*
- ***Glomerulo-tubular.*** *Segment of the proximal tubule to reabsorb a constant fraction of glomerular filtrate and solutes delivered to it.*
- ***Microalbuminuria.*** *It is the urinary albumin excretion of 30–300 mg/day, or 20–200 μg/min) is an earlier sign of vascular damage.*
- ***Nephropathy.*** *This is the deterioration of kidney function.*
- ***Non-albuminuric.*** *No urinary albumin excretion*

Key Facts of Biomarkers of Diabetes-Induced Nephropathy

Diabetic nephropathy is also known as diabetic kidney disease.
It is the chronic loss of kidney function in individuals with diabetes mellitus.
It is one of the leading causes of chronic kidney disease and end-stage renal disease.
A biomarker, or biological marker, is a measurable indicator of some biological state or condition.
Biomarkers of diabetes-induced nephropathy are those biological markers that are used to monitor the status of the kidney in diabetic individuals.

Summary Points

- *Biomarkers of diabetic nephropathy are used to diagnose and predict progression of kidney damage.*
- *Hyperglycemia-induced kidney damage is the main insult responsible for pathological changes in the nephron of diabetic individuals.*
- *Microalbuminuria is the earliest standard routine biomarker to detect diabetic nephropathy in the early stage, but its accuracy is a subject of concerns.*
- *New biomarkers of DN are classified into glomerular damage biomarkers, tubular damage biomarkers, inflammatory biomarkers, and oxidative stress biomarkers based on the source and pathological changes.*
- *Future novel biomarkers of DN with promising diagnostic and prognostic relevance are NOX-4 (isoform of NADPH oxidase), miRNAs, proteomics, metabolomics, and genomics.*

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Serum Vitamin D As a Biomarker in Diabetic: Applications and Associations with Retinopathy

44

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Contents

Introduction 910
Vitamin D Deficiency 911
Vitamin D and Diabetes Mellitus 912
Vitamin D and Diabetic Retinopathy Development and Severity 912
Diabetic Retinopathy Pathophysiology – Where Does Vitamin D Stand? 913
Vitamin D Supplementation for DR Prevention – What Is the Current Evidence? 917
Applications to Prognosis 917
Conclusion 918
Key Facts 918
Mini Dictionary of Terms 918
Summary Points 918
References 919

Abstract

Vitamin D is a multifunctional hormone with multiple biological functions including regulation of endothelial function, angiogenesis, antioxidant and anti-inflammatory pathways. Recent studies have shown that vitamin D plays a role in diabetic retinopathy (DR) pathophysiology. Moreover, it has found that vitamin D deficiency increases the risk of DR development, and that serum levels of vitamin D may correlate with DR severity.

Keywords

Vitamin D · Biomarkers · Diabetes mellitus · Microvascular complications · Diabetic retinopathy

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Abbreviations

AGES	advanced glycation end products
DR	diabetic retinopathy
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes Study
HIF-1	hypoxia-inducible factor-1
IFN-γ	Interferon gamma
IL	interleukin
NLRP3	NLR family pyrin domain containing 3
PDR	proliferative diabetic retinopathy
RAAS	renin-angiotensin-aldosterone system
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF- β	transforming growth factor β
TNF-α	Tumor necrosis factor α
TXNIP	Thioredoxin interacting protein
VD	vitamin D
VDD	vitamin D deficiency
VEGF	vascular endothelial growth factor

Introduction

Vitamin D (VD) is a multifunctional hormone with two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) (Gallieni et al. 2009; Lips 2006). Vitamin D2 is a synthetic molecule and can be found in fortified foods (e.g.,: milk, cereals, bread products) (Gallieni et al. 2009). Vitamin D3 can be obtained from dietary intake but it is also produced in the skin (Gallieni et al. 2009). Vitamin D3 does not have intrinsic biological activity (Lips 2006). Its active compound, 1,25-dihydroxy vitamin D3 (calcitriol), is produced through hydroxylation by the liver and kidney (Bikle 2014). Figure 1 shows the metabolic pathways for VD synthesis.

The active compound of vitamin D3 has diverse biological functions, including calcium and bone metabolism and blood pressure control. Additionally, vitamin D3 plays an important role in endothelial function, angiogenesis, immunoregulation, apoptosis inhibition, and antioxidant pathways (Bikle 2014; Christakos et al. 2016).

Recently, a link between diabetes and Vitamin D deficiency (VDD) has been found in several studies. It was reported that VD alleviates insulin resistance and promotes insulin synthesis and secretion (Bassil et al. 2013). Moreover, an association between VDD and micro- and macrovascular diabetes complications has been established (Joergensen et al. 2011; Ahmed et al. 2020, 2021; Zhao et al. 2021; Herrmann et al. 2015).

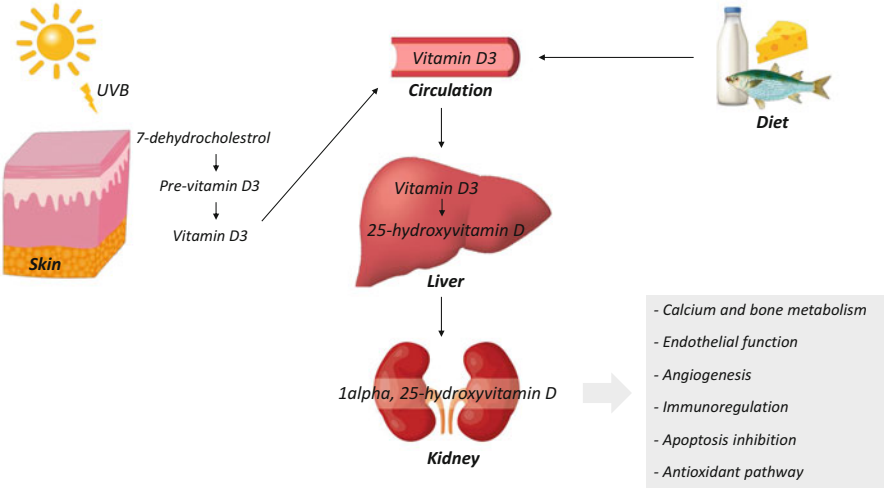

Fig. 1 Overview of the metabolic pathways for vitamin D synthesis

Vitamin D Deficiency

Vitamin D sufficiency is assessed by 25-hydroxyvitamin D (calcidiol) serum measurement. The optimal serum 25-hydroxyvitamin D concentration is still a subject of debate. However, there is agreement among experts that levels below 12 ng/mL represent deficiency and levels above 30 ng/mL are undoubtedly sufficient (Institute of Medicine 2011).

VDD represents a major public health issue, affecting about one billion people worldwide in 2008. Recent large observational studies suggested that about 40% of Europeans have VDD and 13% are severely deficient (Amrein et al. 2020).

The relevance of this widespread deficiency derives from the fact that this condition may affect several body systems, having a major impact in numerous diseases. VDD has been linked to diabetes mellitus, cardiovascular disease, depression, osteoporosis, colorectal and breast cancer, infection, multiple sclerosis, autoimmunity, allergy, and postural instability (Amrein et al. 2020; Wang et al. 2017). In some of this diseases VDD is related with a greater disease severity, morbidity, and mortality (Amrein et al. 2020). However, if VDD only represents a biomarker of diseases severity or if it could be a modifiable risk factor are the main debatable questions.

Vitamin D and Diabetes Mellitus

Several studies have consistently reported that patients with both type 1 (T1DM) and 2 (T2DM) diabetes mellitus patients have consistently lower levels of VD (Daga et al. 2012; Federico et al. 2018; Greer et al. 2013; Rasoul et al. 2016). The pathophysiology is not completely understood, but evidence suggests that VD improves beta-cell function and insulin sensitivity by reducing oxidative damage and by suppressing inflammation and stimulating the insulin signal transduction (Yaribeygi et al. 2020; Mathieu et al. 2005).

Regarding the incidence of DM, several studies have associated low levels of VD with the development of the disease. Lower levels of VD during pregnancy have been associated with a greater risk of T1DM development in the offspring (Sørensen et al. 2012). The long-term prospective Copenhagen City Heart Study found an association between low VD levels and an increased risk of T2DM, showing that the cumulative incidence of T2DM increased with the decreasing VD serum levels (Tsur et al. 2013). Moreover, an inverse association between glycated hemoglobin and VD concentrations has been described (Zoppini et al. 2013).

Additionally, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) Study, confirmed that VDD is a predictor for the development of macro- and microvascular complications in diabetic patients (Herrmann et al. 2015). They found that a 50 nmol/L difference in serum VD was associated with a 23% ($p = 0.007$) increased risk of a macrovascular event (Herrmann et al. 2015).

Vitamin D and Diabetic Retinopathy Development and Severity

Recently, the role of VDD in DR pathogenesis has become a focus of many studies. A positive correlation between the presence and the severity of DR has been described. A meta-analysis of 15 studies confirmed a positive correlation between VDD (defined as VD serum levels below 20 ng/ml) and an increased risk of DR. (Luo et al. 2017) Moreover, VD serum levels and DR severity has been enforced by many studies showing that patients with proliferative DR had a significant lower VD levels than those with nonproliferative DR (Luo et al. 2017).

Moreover, Ahmed et al. studied the association between DR and several VD metabolites and they found that DR was associated with lower 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 concentrations (Ahmed et al. 2021). Additionally, they proposed that the VD metabolite ratio (a ratio of 24,25-dihydroxyvitamin D3/25-hydroxyvitamin D) is a better indicator of VD status (Ahmed et al. 2020). This ratio showed a striking relationship between diabetic complications being lower in patients with DR (Ahmed et al. 2020).

Furthermore, at the genetic level, several polymorphisms in the VD receptor were associated with an increased risk of DR (Dakroury et al. 2020).

Tables 1 and 2 synthetizes the findings of major studies about VD and DR in both T1DM and T2DM.

Table 1 Major studies of diabetic retinopathy and vitamin D in type 1 diabetics

Type 1 Diabetes Mellitus					
Study	Country	Year	Type of study	Sample size	Findings
Joergensen et al. (2011)	Denmark	2011	Prospective	220	No association between vitamin D (VD) levels and diabetic retinopathy (DR)
Lopes et al. (2020)	Portugal	2020	Retrospective	182	Lower levels of VD were associated with an increased prevalence DR
Kaur et al. (2011)	Australia	2011	Cross-sectional	517	DR was associated with VD deficiency (VDD) (odds ratio 2.12 [95% CI 1.03–4.33])
Shimo et al. (2014)	Japan	2014	Report	75	VDD were independent determinant of DR (OR; 3.45, 95% CI; 1.11–10.6, $p = 0.03$)
EURODIAB prospective complications study (Engelen et al. 2015)	16 European countries	2015	Prospective	532	No association was found between VDD and DR

VD, vitamin D; DR, diabetic retinopathy; VDD, vitamin D deficiency

Diabetic Retinopathy Pathophysiology – Where Does Vitamin D Stand?

Classic pathogenetic mechanisms of diabetic microangiopathy includes the pro-angiogenic and pro-inflammatory pathways and recent research has shown that VD can play a role in both (Manuel Falcão and Rocha-Sousa 2010). Figure 2 displays the pathways of DR pathophysiology and the VD mechanisms of action on it.

In the angiogenic pathway, it has been shown that VD is a potent inhibitor of neovascularization in vivo, by downregulating hypoxia-inducible factor-1 (HIF-1) transcriptional activity (Albert et al. 2007). This factor stimulates vascular endothelial growth factor (VEGF) and nitric oxide production, which will induce vasodilation (Tarr et al. 2013). Also VEGF, increases vascular permeability and promotes angiogenesis (Manuel Falcão and Rocha-Sousa 2010). VEGF play a pivotal role in DR genesis and is currently one important therapeutic target for drugs currently used in daily practice (Schmidt-Erfurth et al. 2017).

Table 2 Major studies of diabetic retinopathy and vitamin D in type 2 diabetics

Type 2 Diabetes Mellitus					
Study	Country	Year	Type of study	Sample size	Findings
Nadri et al. (2019)	India	2019	Cross-sectional	72	A cutoff value of 18.6 ng/mL for vitamin D (VD) was found to be significantly associated with proliferative diabetic retinopathy (DR)
Zhao et al. (2021)	Chinese	2021	Cross-sectional	850	VD deficiency (VDD) was not associated with higher risk DR
Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study (Herrmann et al. 2015)	Australia, New Zealand, and Finland	2015	Double-blind, placebo-controlled trial	9,795	Low VD levels are associated with an increased risk of DR
Payne et al. (2012)		2012	Cross-sectional	221	Patients with proliferative DR had low vitamin D levels than those without diabetes
Alcubierre et al. (2015)	Spain	2015	Case–control	283	VDD were associated with the presence of DR; Patients with more severe retinopathy had lower levels of serum VD and had more frequently VDD.
Millen et al. (2016)	US	2016	Prospective	1339	VD concentrations $\geq$75 nmol/L were associated with lower odds of any DR.
Reddy et al. (2015)	Indian	2015	Cross-sectional	164	No association was found between VD and DR
He et al. (2014)	China	2015	Cross-sectional	1520	VDD was associated with increased risk of DR (odds ratio 1.93) and sight-threatening DR (odds ratio 2.42) (both $P < 0.01$)

(continued)

Table 2 (continued)

Type 2 Diabetes Mellitus					
Study	Country	Year	Type of study	Sample size	Findings
Jee et al. (Jee et al. 2014)	Korea	2014	Cross-sectional	2113	Inverse relationship between VD levels and any DR and proliferative DR only in men
Long et al. (2017)	China	2017	Cross-sectional	842	Association between VDD and DR severity only found in patients with well-controlled glycemia
Zoppini et al. (2013)	Italy	2015	Cross-sectional	715	Serum VD levels decreased significantly in relation to the severity of DR
Ahmed et al. (2020)	Qatar	2020	Cross-sectional	460	1,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 levels were associated with DR

VD, vitamin D; DR, diabetic retinopathy; VDD, vitamin D deficiency

Many DR features are intimately linked to inflammatory processes such as tissue edema, increased blood flow, and inflammatory cells migration. VD downregulates inflammation in the retina (Christakos et al. 2016; Tecilazich et al. 2020). Specifically, VD might decrease immunocyte proliferation and the expression of proinflammatory cytokines (Mathieu et al. 2005). VD also decreases the activation of helper T-cells, cytotoxic T-cells, natural killer cells, macrophages, and dendritic cells in the retina (Palomer et al. 2008). Moreover, patients with PDR and VDD had an increased production of Interferon gamma (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-17. VD inhibits peripheral blood monocular cells, resulting in reduced levels of the aforementioned cytokines (Yi et al. 2016).

Lu et al. showed that VD decreases ROS production, thus downregulating thioredoxin interacting protein (TXNIP) expression and blocking the activation of NLR family pyrin domain containing 3 (NLRP3), which reduces apoptosis and vascular permeability (Lu et al. 2018). Also, this eventually could downregulate IL-6, decreasing the retinal pro-inflammatory environment of DR. (Lu et al. 2018) The NLRP3/IL-1β pathway plays a critical role in the development and progression of DR. (Lu et al. 2018)

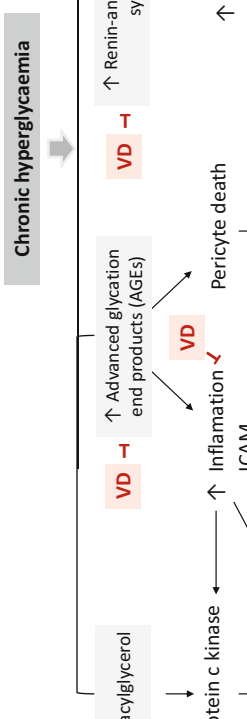

Fig. 2 Vitamin D role in diabetic retinopathy physiopathology. VEGF, vascular endothelial growth factor; HIF-1, hypoxia inducible factor-1; ICAM, intercellular adhesion molecule 1; AGES, advanced glycation end products; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species

The role of VD is also observed in the complement pathway. By modulating liver inflammation and C3 adipocytes secretion, VD reduces circulating C3 (Abbas 2017). In DR, fragments of complement can bind to their receptors on retinal cells, leading to inflammation or synthesis of angiogenic growth factors (Chrzanowska et al. 2018).

Several growth factors have been linked with DR pathogenesis, including TGF- β (transforming growth factor β) (Tarr et al. 2013). Diabetic rats receiving VD supplementation showed lower levels of TGF-β in the retina, when compared with diabetic controls (Ren et al. 2012).

The renin-angiotensin-aldosterone system (RAAS) is hyperactive in DR. It has been reported that the retinal angiotensin receptors are increased in patients with PDR (Tarr et al. 2013). It was hypothesized that an increase in VD serum levels may decrease renal secretion of renin and decrease plasma renin activity (Burgess et al. 1990).

Advanced glycation end products (AGEs) result from chronic hyperglycemia and have been correlated with DR onset and severity (Manuel Falcão and Rocha-Sousa 2010). Also, AGES may lead to ROS production, VEGF production, and vascular hyperpermeability (Manuel Falcão and Rocha-Sousa 2010). In T2DM, VD supplementation decreases AGES levels, which could potentially delay or prevent DR development (Omidian et al. 2019).

Vitamin D Supplementation for DR Prevention – What Is the Current Evidence?

The effects of VD supplementation on the risk of developing either micro- or macrovascular complications of DM are currently poorly studied. To date there is no scientific foundation to give vitamin D supplementation to prevent DR development in diabetics.

However, a meta-analysis assessing vitamin D supplementation effects on inflammatory risk factors showed that supplementation significantly decreased protein C reactive levels, but did not affect TNF-α or IL-6 (Yu et al. 2018). Since inflammation is an important part of DR pathophysiology and this study shows a decrease in some inflammatory biomarkers, vitamin D supplementation might have a role in patient treatment. More studies in this field are needed to address this question.

Applications to Prognosis

VD could be a biomarker for both DR development and progression. The relationship between VD and DR was found in several studies as mentioned previously. However, caution is needed. VD levels have several confounders such as physical activity, sunlight exposure, diet, and reverse causality. The implications of these confounders are currently unknown.

Conclusion

VDD and DR are two widespread conditions, with a great impact on public health. Recently, there is growing evidence that VD deficiency increases the risk of DR development and its severity, making VD a potential usable biomarker in daily practice. However, at this point, more studies about VD supplementation in diabetics and its impact in DR prevention and treatment are still needed.

Key Facts

- Low vitamin D (VD) levels increase the risk for diabetic retinopathy (DR) development.
- VD levels correlate with DR severity, specifically patients with proliferative DR have lower VD serum concentrations than those without proliferative DR.

Mini Dictionary of Terms

Diabetic Retinopathy: Vascular retinopathy caused by long-standing any type of uncontrolled diabetes mellitus. A major cause of blindness.

Proliferative Diabetic Retinopathy: An advanced stage of diabetic retinopathy in which new vessels are formed from preexisting retinal vessels when there is significant retinal ischemia. Leads to vitreous hemorrhages or tractional retinal detachments.

Diabetic Macular Edema: Presence of extra-cellular fluid in the foveal area that leads to poor retinal function and central visual loss.

Vascular Endothelial Growth Factor: Major cytokine in the pathogenesis of diabetic retinopathy. Is implicated in both proliferative diabetic retinopathy and diabetic macular edema.

Vitamin D deficiency: Low levels of circulating vitamin D. May be involved in the development and progression of different stages of diabetic retinopathy.

Summary Points

- Vitamin D (VD) has multiple biological functions including regulation of endothelial function, angiogenesis, antioxidant, and anti-inflammatory pathways.
- VD plays a role in diabetic retinopathy (DR) pathophysiology.
- Vitamin D deficiency increases the risk of DR development.
- Low VD levels correlates with DR severity.
- To date there is no scientific foundation to supplement vitamin D to prevent DR development in diabetic patients.

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Markers of Bacterial Translocation in Type 2 Diabetes Mellitus

45

Marwa Ahmed Meheissen

Contents

Introduction 925
Gut Microbiome 925
The Gut Barrier's Defense Mechanisms 927
The Gut Barrier Is Composed of Multiple Layers: (Fig. 1) 927
Factors Affecting the Gut Barrier Integrity 930
Mechanisms of Gut Barrier Disruption 931
Consequences of Gut Barrier Disruption/Dysfunction 932
Bacterial Translocation 933
Markers of Bacterial Translocation 935
  Lipopolysaccharide (LPS) 935
  Bacteria and Bacterial DNA 936
  Other Bacterial Translocation Markers 937
Applications to Other Diseases/Conditions 937
Mini-Dictionary of Terms 941
Key Facts 941
Summary Points 941
References 942

Abstract

Type 2 Diabetes mellitus is a multifactorial metabolic disorder. The growing body of evidence linking the gut microbiome to host metabolism has been accompanied by change in the study of metabolic illnesses including type 2 diabetes. Disturbance in the balance of gut microbiome "gut dysbiosis" has led to the emergence of the concept of "leaky gut" and metabolic endotoxemia. Previous studies observed higher lipopolysaccharide or lipopolysaccharide binding protein

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concentrations in diabetics than in healthy controls. Translocation of bacteria and their products through the disrupted gut barrier increased the circulation of markers that could be linked to increased intestinal permeability. The end result of translocated products is the stimulation of systemic inflammatory response affecting many organs and increasing insulin resistance thus aggravating diabetes. It is anticipated that by gaining a better understanding of the mechanism of leaky gut and bacterial translocation in diabetes, scientists will be able to develop novel diagnostic and therapeutic approaches.

Keywords

Type 2 diabetes mellitus · Gut barrier · Intestinal permeability · Microbiome · Gut dysbiosis · Lipopolysaccharide · Endotoxin · Bacterial translocation · Endotoxemia · Toll-like receptor · Inflammatory cytokines

Abbreviations

AHR	Aryl hydrocarbon receptor
AMPs	Antimicrobial peptides
COVID-19	Coronavirus Disease 2019
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EndoCAb	Endotoxin core antibodies
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
IAP	Intestinal alkaline phosphatase
IL	Interleukin
INF-γ	interferon-gamma
LAL	Limulus Amoebocyte Lysate
LDL-R	Low density lipoprotein receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear Factor Kappa B
PAMPs	Pathogen associated molecular patterns
PG	Peptidoglycan
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome Coronavirus 2
SCFAs	Short chain fatty acids
sIgA	Secretory immunoglobulin A
T2DM	Type 2 diabetes mellitus
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
16SrRNA	16S ribosomal ribonucleic acid

Introduction

Diabetes is a complicated metabolic condition caused by a combination of factors including genetics, sex, lifestyle, diet, environmental, and epigenetics. Diabetes has become more common as living standards have improved and people's lifestyles have changed. Over 422 million people worldwide suffer from type 2 diabetes mellitus (T2DM), which accounts for 90% of all diabetes cases (Cho et al. 2018).

It was shown that obesity is a high-risk factor for T2DM. The main characteristics of T2DM are hyperglycemia and insulin resistance, and most importantly the chronic inflammatory state throughout diabetes progression. All these factors are involved in a number of body disorders, and have a negative impact on several organs like cardiovascular diseases, diabetic retinopathy, and diabetic nephropathy (Donath and Shoelson 2011).

The study of the mechanisms behind metabolic disorders like T2DM, obesity, and related cardiovascular comorbidities has progressed significantly. Microbiome research and gut dysbiosis with its consequences of leaky gut have recently gained interest for diagnostic and therapeutic purposes (Chakaroun et al. 2020).

While interaction between the gut microbiome and peripheral organs including the brain, liver, adipose tissue, muscle, and pancreas has emerged as an important element for homeostatic systems, the quest for communication routes has resurrected the concept of "leaky gut." This concept is predicated on the idea of bacterial translocation where complete bacteria, as well as bacterial products such as metabolites, being translocated into the circulation and distant tissues, contributing to metabolic disease-related remote organ harm (Chakaroun et al. 2020).

The loss of the gut barrier is a major contributor to metabolic (local and systemic) inflammation in the body. Intestinal barrier defects have been linked to a variety of diseases, including gastrointestinal (inflammatory bowel disease, colon cancer), as well as extra-intestinal (diabetes, obesity). All of these disorders are thought to be caused by a malfunction of the intestinal barrier and an unregulated flow of antigens across the intestinal wall that may challenge the immune system of susceptible individuals triggering inflammatory mechanisms in the GIT or other organs (Vancamelbeke and Vermeire 2017).

Gut Microbiome

The microbiome is a dense and diverse microbial population that includes archaea, bacteria, protozoans, and viruses and is found in the gastrointestinal tract (GIT). There are roughly 100 trillion bacteria that populate the gut mucosal surface, continually interacting with immunologically and metabolically active cells (McDermott and Huffnagle 2014).

Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia, which make up the bulk of the gut microbiota, play an important role in defending the host against pathogenic microbes. The intestinal microbiota encodes around 3.3

million genes, with over 1,000 bacterial species recognized as part of this ecosystem (Qin et al. 2010).

These bacteria not only act as a first line of defense against foreign antigens, but they also improve mucosal and systemic immunity. Complex carbohydrate digestion, vitamin synthesis, immunological and inflammatory response control, and hormone and neurotransmitter generation are only a few of the physiological roles of the gut microbiota (Fan and Pedersen 2021).

Since dietary fibers cannot be digested by our gastrointestinal system, they are fermented by the gut microbiota, which produce short-chain fatty acids (SCFAs) as metabolites. SCFAs bind to G-protein coupled receptors, stimulate the production of glucagon-like peptide-1, a critical incretin hormone produced by enteroendocrine L cells. Glucagon-like peptide-1 (GLP-1) inhibits glucagon secretion, inhibits gluconeogenesis in the liver, improves insulin sensitivity, and increases central satiety, all of which lead to weight loss and better control of diabetes. SCFAs can also prevent bacteria from migrating from the intestines into the mesenteric adipose tissue and circulation, thus inhibit the low-grade inflammatory response. It was found that in persons with T2DM, the number of microorganisms involved in SCFA synthesis is much decreased (Amar et al. 2011a).

Various signaling channels transmit the multiple interactions between gut microbiota-derived metabolites, the gut microbiota, and the host immune system. The direct chemical communication between gut bacteria, the host, and immune pathways is referred to as the host–microbe metabolic axes. These biological signals have a system-wide impact and have direct effects on different body organs. Gut microbiota drive metabolic responses within these axes, and both the gut microbiome and host genome produce choline, phenols, bile acids, and SCFAs, which are essential for health. These complex interactions between the gut microbiome and its host are critical for sustaining good health and may be linked to the emergence of illnesses like diabetes (Aw and Fukuda 2015).

The microbiome's composition is influenced by a variety of factors. Patients with T2DM have a different gut microbiome composition than healthy people. The exact gut microbiota signatures of diabetes problems, on the other hand, have yet to be discovered. Qin et al. (2012) used a metagenome-wide-association-study approach to evaluate the gut microbial metagenome data of 345 T2DM patients and non-diabetic controls of Chinese individuals, and found that microbiome in T2DM group were mostly opportunistic pathogens like *Bacteroides caccae, Clostridium hathewayi, Clostridium ramosum, Clostridium symbiosum, Eggerthella lenta*, and *E. coli*, as well as mucin-degrading microbes: *Akkermansia muciniphila*, and sulfate-reducing microbes: *Desulfovibrio spp*. The non-diabetes control group, on the other hand, had practically microbiome from butyrate-producing bacteria, such as *Clostridiales spp., Roseburia intestinalis, and Roseburia intestinalis and Faecalibacterium prausnitzii*.

In a European cohort (Karlsson et al. 2013), however, the results were quite different. In the European study, obese people with less severe metabolic syndrome had higher *Akkermansia muciniphila* abundances, which was linked to greater microbial diversity as compared to those who were metabolically compromised.

These findings suggest that the link between *Akkermansia muciniphila* and T2DM may be population-based.

It was also found that in human male T2DM patients, there were much less Firmicutes, including Clostridia, compared to non-diabetic healthy patients. The ratios of Bacteroidetes to Firmicutes and the Bacteroides–Prevotella group to the Clostridium coccoides–Eubacterium rectale group were positively correlated with plasma glucose levels. Furthermore, T2DM patients had higher levels of Betaproteobacteria than controls. These findings suggest that Gram-negative Proteobacteria and Bacteroidetes might contribute to the pathogenesis of T2DM through an endotoxin-induced inflammatory response, since the endotoxin lipopolysaccharide (LPS) is found in large proportions as a major component of the outer membrane of gram-negative bacteria (Larsen et al. 2010). Moreover, gut metagenome-based computational models may predict T2DM associated phenotype in glucose-intolerant patients, suggesting that the gut microbiota could be a novel biomarker for T2DM prediction (Karlsson et al. 2013).

The Gut Barrier's Defense Mechanisms

The GIT is exposed to thousands of microorganisms and nutrient components as a result of food ingestion. Gut homeostasis thus necessitates a sophisticated system capable of executing a variety of tasks, including the elimination of bacterial endotoxins, hindering direct contact with pathogens, and regulating absorption of nutrients while preventing hazardous substances or bacteria from being transported, promoting an immune response, and reducing proliferation of pathogenic bacteria (Ghosh et al. 2020).

The Gut Barrier Is Composed of Multiple Layers: (Fig. 1)

1. The inner most layer containing the intestinal alkaline phosphatase (IAP), considered the functional luminal first line of defense that dephosphorylates bacterial LPS reducing local intestinal inflammation. LPS, a component of gram-negative cell wall, is associated with systemic inflammation through the Lipid-A moiety which binds to toll-like receptor-4 (TLR4). Removal of one phosphate group on the Lipid-A moiety reduces LPS toxicity several fold by decreasing downstream intracellular signaling, resulting in decreased Nuclear Factor Kappa B (NF-κB) activation and cytokine release. IAP also modulates bicarbonate secretion, duodenal surface pH, and long chain fatty acid absorption. In addition, it dephosphorylates adenosine di- and tri-phosphate (ADP & ATP); high levels of ATP suppress the growth of gut bacteria and alter bacterial homeostasis (Lallès 2014; Malo et al. 2014; Ghosh et al. 2020).
2. the mucus layer which is considered the first physical barrier hindering interactions between gut microbiota and epithelial cells. It is made up of two layers: an inner layer that is tightly linked to the epithelial cells and an outer layer that is

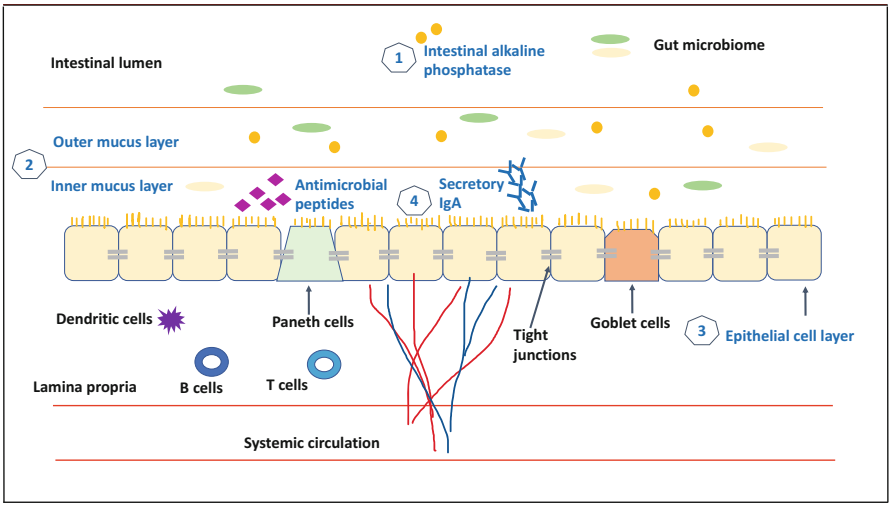

Fig. 1 The layers of the gut barrier. 1. Intestinal alkaline phosphatase: functional barrier that dephosphorylates luminal lipopolysaccharide, rendering it inactive. 2. Mucus: physical barrier consisting of outer and inner layer, prevents luminal bacteria from interacting with epithelial cells. 3. Epithelial cell layer: physical barrier separates intestinal lumen from systemic circulation, containing goblet cells which secretes mucin and Paneth cells which secretes antimicrobial peptides. 4. Functional layer consisting of antimicrobial peptides and secretory IgA (produced by B cells from the lamina propria). (Modified from Ghosh et al. 2020)

thicker but less adherent. The inner mucosal layer prevents direct contact between epithelial cells and bacteria. While, the outer layer carries commensals that prevent the entry of pathogenic bacteria. MUC2 is a highly glycated mucin glycoprotein released by specialized epithelial cells known as goblet cells. MUC2 forms an ordered mucous layer after secretion, producing a hydrated and extended network with other released proteins. The amount and type of mucus in the mucosa is a balance between mucus secretion and bacterial erosion and breakdown of the mucin layer. Therefore, probiotic or prebiotic supplements that enhance the amount of mucus-residing commensal bacteria are expected to improve the mucosal layer's barrier function (Deplancke and Gaskins 2001; Ghosh et al. 2020).

3. The intestinal epithelium and the intercellular tight junctions which prevent transport of bacteria or their products to systemic circulation. The single layer intestinal epithelium performs a selective transport function where it allows the passage of nutrients, water, and electrolytes, while prevents the passage of pathogens, their toxins and antigens from inside the lumen to the systemic circulation. Its function is controlled by transcellular and paracellular transport pathways which is mediated by intercellular complexes (desmosomes, adhesive junctional, tight junctional) localized at the apico-lateral membrane junction and along the lateral membrane. Desmosomes and adhesive junctional proteins are transmembrane proteins which link adjacent cells to the actin cytoskeleton.

The paracellular transport is controlled by tight junctions (occludins, claudin family proteins) which seal the intercellular space. Furthermore, due to abnormally high levels of glycolipids arranged in lipid raft microdomains stabilized by divalent galectin-4, the intestinal epithelium is regarded nonpermissive for endocytic uptake. Despite the crucial contributions of the other three layers, the integrity of the epithelial cell layer and tight junction proteins is generally referred to be the gut barrier (Anderson 2001; Ghosh et al. 2020).

4. The outer most layer consisting of antimicrobial proteins and secretory immunoglobulin A (sIgA). Paneth cells, intestinal secretory cells at the base of intestinal crypts, play a major role in innate immunity by secreting antimicrobial peptides that play a significant role in the host defense against gut bacteria. Alpha defensin, a member of the defensing family of peptides, is the most prevalent antimicrobial peptide in the human intestine, that are bactericidal to both Gram-positive and negative bacteria. sIgA is produced by plasma of the lamina propria. It binds to microbes or toxins preventing colonization or damage of epithelial cells. It also binds and clears immune complexes in the lamina thus reducing systemic inflammatory responses (Mestecky et al. 1999; Ghosh et al. 2020).

In the intestine, dendritic cells (DCs) play a pivotal role to extract antigens from intestinal pathogens, and to release cytokines such as IL-17 and IL-22 and to increase the expression of antimicrobial peptides (AMPs). Chemokines and inflammatory markers in turn recruit neutrophils from blood vessels into the intestinal tract to fight pathogenic microorganisms. NLRP6 (NOD-like receptor family pyrin domain containing 6) is triggered by microbial metabolites in intestinal epithelial cells, increasing IL-18 and AMPs and therefore defending the intestinal barrier (Hooper et al. 2012) (Fig. 2).

Polyphenols (present in fruits, vegetables, and cereals) are converted to aryl hydrocarbon receptor (AHR) ligand by gut microbiota, which promotes the production of intestinal tight junction proteins and probiotic growth (Yang et al. 2021). Tryptophan metabolites of indole derivatives, can directly activate the AHR on intestinal immune cells, facilitating the production of IL-22, antigenic peptides, mucin, and tight junction proteins and thus protecting the intestinal barrier. By prompting L cells to release glucagon-like peptide-1 (GLP-1), indole and indoleacetic acid can also speed up insulin secretion (Hendrikx and Schnabl 2019) (Fig. 2).

Short-chain fatty acids (ScFAs) including acetate, propionate, and butyrate are produced by some gut bacteria from the fermentation of undigested and unabsorbed carbohydrates. ScFAs have a substantial impact on the health of GIT wall by acting as a source of energy, anti-inflammatory, vasodilator, and angiogenic (Ibrahim et al. 2011). Butyrate supports mucosal barrier integrity via modulating intestinal epithelial proliferation, apoptosis, and cell differentiation, as well as inhibiting NF-κB (Ogawa et al. 2003). Butyrate supplementation reduced body adiposity, insulin resistance, hyperinsulinemia, and hyperglycemia in obese, prediabetic mice. It also improved the intestinal epithelial barrier and insulin secretion from beta cells (Matheus et al. 2017).

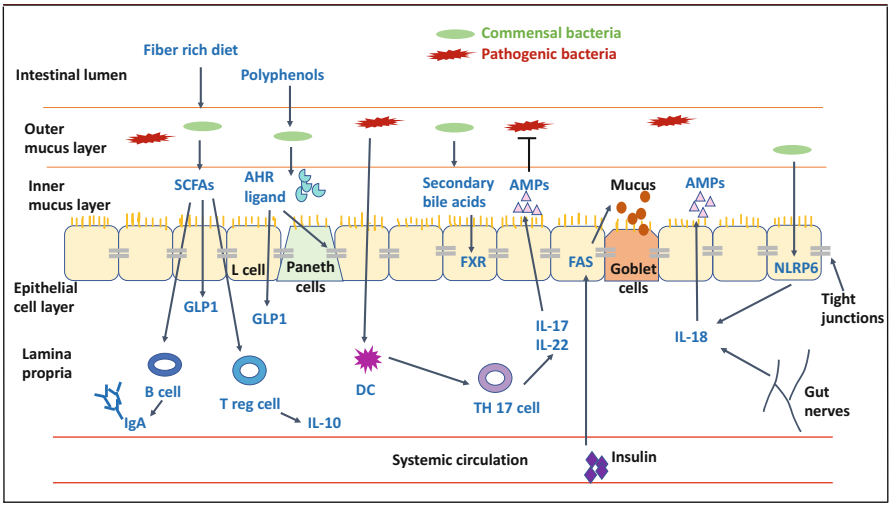

Fig. 2 The gut barrier defense mechanisms. DCs play a pivotal role to extract antigens from intestinal pathogens, and to release cytokines such as IL-17 and IL-22 and to increase the expression of AMPs. NLRP6 is triggered by microbial metabolites, increasing IL-18 and AMPs. Polyphenols converted to AHR ligand by gut microbiome, which promotes the production of intestinal tight junction proteins and stimulates L cells to release GLP-1 speeding up insulin secretion. SCFAs activate B cells, causing them to produce antibodies as well as T-regulatory cells boosting IL-10 secretion. Secondary bile acids produced by gut microbiome preserve the integrity of the intestinal barrier using the FXR pathway. Insulin molecules increase the expression of FAS helping mucus secretion. The gut nerves can also directly stimulate IL-18 and AMPs production maintaining gut barrier's integrity. (AHR: aryl hydrocarbon receptor, AMPs: Antimicrobial peptides, DCs: Dendritic cells, FAS: fatty acid synthase, FXR: farnesoid X receptor, GLP-1: glucagon-like peptide-1, IL: interleukin, NLRP6: NOD-like receptor family pyrin domain containing 6). (Modified from Yang et al. 2021)

SCFAs can also activate B cells, causing them to produce more antibodies. The increased number of T-regulatory cells triggered by SCFAs helps boosting the secretion of IL-10 (anti-inflammatory cytokine), and assisting gut bacteria colonization. Secondary bile acids produced by gut bacteria can also preserve the integrity of the intestinal barrier using the farnesoid X receptor pathway (Yang et al. 2021) (Fig. 2).

Furthermore, insulin molecules increase the expression of fatty acid synthase in intestinal epithelial cells, which aids mucus secretion. The gut nerves can also directly stimulate IL-18 and AMPs production that helps maintaining gut barrier's integrity (Yang et al. 2021) (Fig. 2).

Factors Affecting the Gut Barrier Integrity

A complex interaction of environmental factors, nutritional factors, antibiotics, and microbiome alteration, as well as other endogenous factors can modify the permeability of gut barrier directly or indirectly (Massier et al. 2021).

Unhealthy eating habits, such as a high-sugar, high-fat, low-fiber western diet, food additives change the composition of the gut microbiota, encouraging pathogen growth while preventing beneficial bacteria. Germs can break down intestinal mucus and penetrate the epithelial barrier (Wu et al. 2011; Zinöcker and Lindseth 2018).

Continuous epithelial cell shedding under several conditions that allows opening of tight junctions and translocation of bacteria, their DNA, metabolites, and LPS such as bacteria or their nucleic acids, metabolites, toxins, and lipopolysaccharides from the intestine to the systemic circulation (Sato et al. 2009).

Increased proinflammatory cytokines like interferon-gamma (INF-γ) and tumor necrosis factor alpha (TNF-α) disrupt the homeostatic balance and allow paracellular transport by downregulation of claudin-1 expression (Hu et al. 2013).

Mechanisms of Gut Barrier Disruption

The breakdown of the intestinal barrier in metabolic illnesses like T2DM is caused by a combination of endogenous and exogenous variables, with dietary factors having the most direct effect. In addition to causing obesity, high-fat and high-fructose diets, as well as dietary additives, cause gut microbiota dysbiosis, and increased intestinal permeability (Cani et al. 2007; Cani et al. 2008; Sohet et al. 2009). As a result, bacteria, pro-inflammatory cytokines, toxins, and metabolites are able to pass easily to the circulatory system through the gut barrier (Cani et al. 2007; Cani et al. 2008; Desai et al. 2016). Hyperglycemia has been observed to impair the integrity of TJ and adhesive connections in T2DM patients in a two-way glucose transporter (GLUT2)-dependent manner, as well as speed up the destruction of the intestinal barrier (Thaiss et al. 2018).

Gut dysbiosis plays a major role in the degradation of the gut barrier in T2DM and related diseases, according to several studies. *Bacteroides caccae*, common in low-fiber diets, secretes a number of mucus-degrading proteases that can destroy the colon's mucus layer (Desai et al. 2016). The amount of bacteria that protect the gut barrier, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, is reduced in T2DM and non-alcoholic fatty liver disease (NAFLD), whereas *E. coli* is raised and can produce the metabolic enzyme StcE to lyse mucin and disrupt the gut barrier (Grys et al. 2005). Bacteroidetes and Firmicutes boost ATP-binding cassette transporter expression and glucagon-like peptide 1 and 2 secretion in enterocytes (Sohail et al. 2017). Everard et al. (2011) discovered a link between gut permeability, glucose intolerance, blood triglyceride, and *Anaerotruncus* abundance in the GIT.

Furthermore, in high-fat diet animals, antibiotics can be used to eliminate the gut microbiota, resulting in intestinal barrier protection (Cani et al. 2008). On the other hand, Lactobacillus supplementation improved insulin homeostasis, glucose tolerance, and beta cell islet protection in diabetic mice (Li et al. 2017).

Consequences of Gut Barrier Disruption/Dysfunction

The disruption or dysfunction of gut barrier is linked to local and systemic inflammatory effects, primarily due to direct interaction of bacteria/bacterial products with epithelial cells and their translocation to the systemic circulation (Ghosh et al. 2020). The disrupted gut barrier allows intestinal bacteria and their pathogen associated molecular patterns (PAMPs), such as LPS and peptidoglycan, as well as other bacterial metabolites to enter the bloodstream. LPS of Gram-negative bacteria passes through the gut mucosal barrier by penetrating tight junctions or chylomicrons producing metabolic endotoxemia. This is thought to be the source of T2DM and other metabolic illnesses' systemic chronic inflammation (Cani et al. 2007).

It's worth mentioning that some bacteria are more likely to translocate than others, which is linked to their ability to bypass or weaken host defensive mechanisms. Gram-negative bacteria including *E. coli* and other *Enterobacterales* and *Enterococci* have been reported to translocate more frequently than other gut bacteria. Nonetheless, inflammation or metabolic stress may affect bacterial translocation rates by increasing permeability to particular taxa or driving active bacterial translocation (Chakaroun et al. 2020).

Direct contact with bacteria/bacterial products activates immune cells in the lamina propria via TLR4/MyD88-dependent signaling pathways through binding to LPS, ending by the production of proinflammatory mediators that prolong local inflammation. The enhanced paracellular transfer of LPS into systemic circulation is, however, the most serious consequence of gut barrier disruption. LPS is transported in the bloodstream attached to either LPS binding protein (LBP) or lipoproteins, where it interacts with immune cell surface receptors (e.g., TLR4), triggering an inflammatory response. TLR4 cannot bind LPS on its own; it requires CD14 as a cofactor, which aids in the transport of LPS to TLR4 and the modulation of LPS recognition by MD2. LPS is shuttled to CD14 by the LPS binding protein. The signal is triggered by the association of these auxiliary molecules, which results in TLR4 homodimerization and intracellular signaling via MyD88 (Lu et al. 2008; Ghosh et al. 2020) (Fig. 3).

This triggers the activation of NF-κB, which leads to increased transcription of proinflammatory cytokines such TNF-α, IL-1, and IL-6 (Akira, Takeda 2004). Tissue inflammation is caused by the infiltration of activated macrophages or the direct activation of resident macrophages in peripheral tissues by circulating LPS. More immune cells (including neutrophils and monocytes) infiltrate the tissue in response to the proinflammatory environment, perpetuating the inflammation and disrupting tissue homeostasis. Diabetes is caused by increased adipose tissue or skeletal muscle inflammation/insulin resistance (Ghosh et al. 2020) (Fig. 3).

Increased expression of TNF-α, IL-1, and IL-6 is linked to the development of insulin resistance and T2DM in obese people. T2DM patients who were given IL-1 receptor antagonists showed a significant reduction in hyperglycemia, with insulin secretion being stable for at least 39 weeks (Böni-Schnetzler et al. 2018).

Furthermore, the metabolites of the gut microbiota can infiltrate the circulatory system and affect distant organs such as the liver, kidneys, adipose tissue, and the

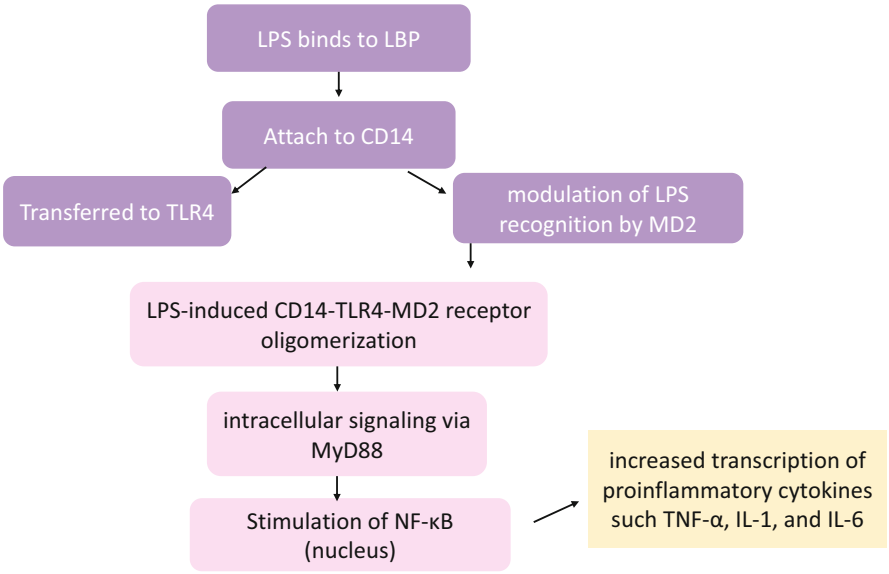

Fig. 3 LPS mechanism of action to induce metabolic endotoxemia. CD14: cluster of differentiation 14, IL: Interleukin, LPS: Lipopolysaccharide, LBP: Lipopolysaccharide binding protein, NF-κB: Nuclear Factor Kappa B, TLR: Toll-like receptor, TNF: Tumor necrosis factor

cardiovascular system by passing through the gut barrier. As a result, they can influence the progression of T2DM (Yang et al. 2021). LPS that enters the liver via the portal circulation activates local macrophages or Kupffer cells, which leads to enhanced hepatic insulin resistance and lipogenesis. Activated macrophages penetrate adipose tissue, induce inflammation and insulin resistance. Insulin resistance develops also in skeletal muscles due to increased inflammation. Foam cell generation is triggered by an activated macrophage infiltration into the arterial wall, which leads to atherosclerotic plaque formation (Ghosh et al. 2020) (Fig. 4).

Bacterial Translocation

Bacterial translocation is the movement of live bacteria from the intestinal lumen to the mesenteric lymph nodes and other locations. Later, the definition of bacterial translocation was expanded to include microbial products or fragments such as lipopolysaccharide (endotoxin), peptidoglycan (PG), lipopeptides, and bacterial DNA (Fukui 2015).

Through disturbed gut tight junctions, microbes and microbial compounds are translocated from the intestinal lumen to the circulation. They trigger inflammation in diverse organs by reacting to TLRs in macrophages, lymphocytes, and other cells. Every TLR recognizes a specific PAMP; endotoxin/LPS (recognized by TLR4), bacterial lipoprotein and PG (recognized by TLR2), flagellin (recognized

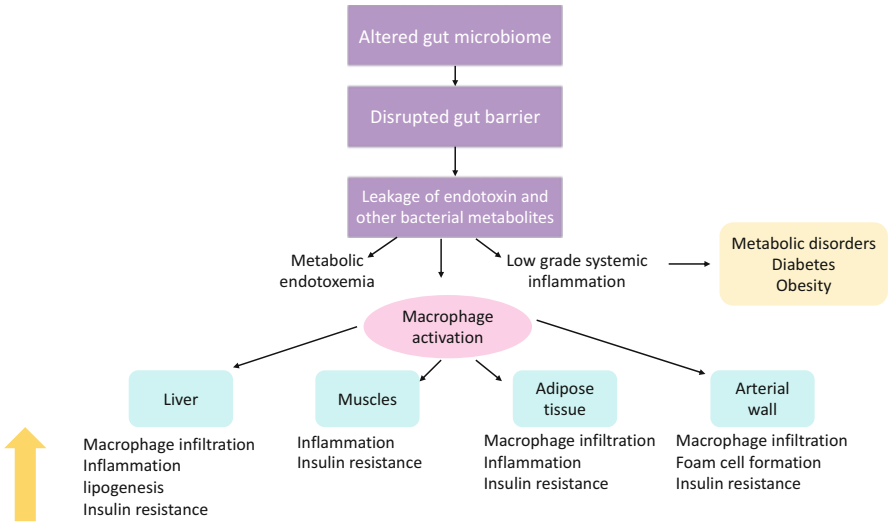

Fig. 4 Consequences of disruption of intestinal barrier in type 2 diabetes mellitus

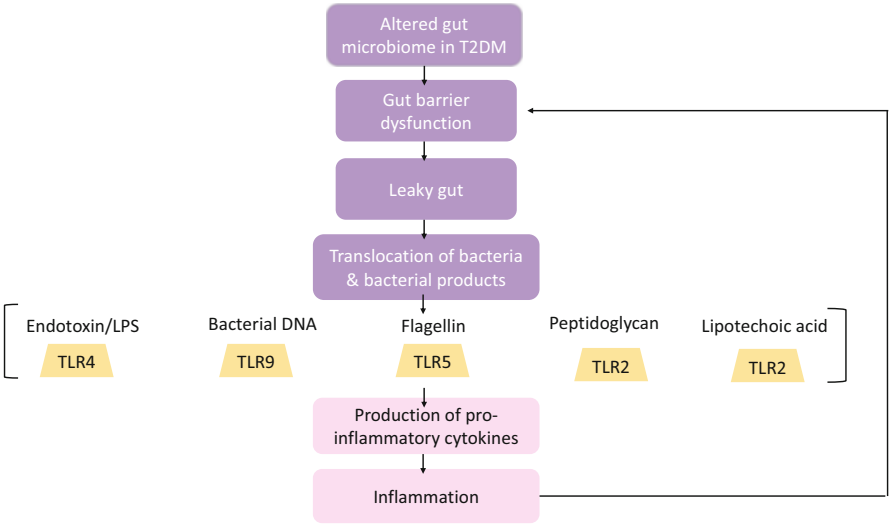

Fig. 5 Mechanism of action of different bacterial translocation markers. LPS: Lipopolysaccharide, TLR: Toll-like receptor, T2DM: Type 2 diabetes mellitus

by TLR5), unmethylated DNA (recognized by TLR9), double-stranded RNA (recognized by TLR3), and single-stranded RNA (recognized by TLR7, TLR8) are among the pathogen-associated mole (detected by TLR7 and 8) (Fukui 2016) (Fig. 5).

Markers of Bacterial Translocation

Lipopolysaccharide (LPS)

Increased intestinal permeability can be indirectly verified by measuring bacterial components in the circulation. This link was discovered after it was shown that sepsis is linked to an acute but reversible state of insulin resistance (White et al. 1987). To this purpose, the majority of research have concentrated on measuring LPS, or endotoxins, which are chemicals found on the outer membrane of gram-negative bacteria. "Metabolic endotoxemia" refers to an increased exposure to bacterial LPS in the blood as a result of obesity or metabolic illness (Massier et al. 2021).

LPS that is taken up from the gut due to increased intestinal permeability is likely to enter the portal vein and be delivered to the liver in the first phase, where it is quickly eliminated. After uptake by various lipoprotein receptors, such as LDL-R (Topchiy et al. 2016), clearance is mostly completed in the liver by hepatocytes and Kupffer cells, and is then excreted to the bile. Furthermore, because LPS binding to lipoproteins and chylomicrons has been demonstrated to inhibit endotoxin-induced monocyte activation and proinflammatory cytokine production (Cavaillon et al. 1990; Harris et al. 1993), the redistribution and increased content of phospholipids among distinct lipoproteins has been proposed as a possible method for attenuating LPS' immunostimulatory effects (Massier et al. 2021).

Endotoxin measurement, especially at low concentrations, is difficult and error-prone, leading to much debate about the test's significance and interpretation. One important point to keep in mind is that LPS is not actively excreted by bacteria, but rather released after gram-negative bacterial cell death and lysis, which does not explain the widespread use of LPS as a surrogate marker for "live" bacterial translocation. However, there may be some intrinsic value in increasing the host's exposure to bacteria in general. The Limulus Amoebocyte Lysate (LAL) assay has been used to measure LPS, ELISA kits are also available, however, not standardized (Massier et al. 2021).

LPS is detectable in low amounts in healthy individuals, and a single meal rich in fat content raises LPS levels (Harte et al. 2012). Many large cohort studies have found elevated levels of LPS and LBP in people with metabolic syndrome or T2DM (Lassenius et al. 2011; Pussinen et al. 2011; Cox et al. 2017). Eight-week overfeeding interventional study was linked to increased endotoxemia, implying a relationship between overnutrition and insulin resistance (Krogh-Madsen et al. 2008). Furthermore, LPS treatment in vitro has been demonstrated to increase intestinal permeability by causing tight junction dysfunction via a TLR4-dependent process (Guo et al. 2015). Cox et al. (2017) calculated a permeability risk score using LPS, LBP, and intestinal fatty acid binding protein (iFABP), which was higher in people with T2DM. In terms of the effects of metabolic intervention on LPS, gastric bypass surgery has been demonstrated to reduce LPS levels by 20% in patients with obesity and T2DM following a 180-day follow-up period (Monte et al. 2012), which was also confirmed for sleeve gastrectomy and duodenal switch surgeries (Trøseid et al. 2013; Clemente-Postigo et al. 2015).

Despite its widespread use, the use of LPS as an intestinal permeability marker has significant drawbacks. These are connected in part to the fact that the requirement for bacterial death/lysis for LPS release makes LPS measurement as a proxy marker for "live" bacterium translocation problematic. Several preanalytical difficulties, such as the lack of sampling in pyrogen-free tubes or the need for sample pretreatment to overcome low LPS recovery, have contributed to the broad range variation of LPS levels reported in the literature (Lassenius et al. 2011; Chakaroun et al. 2020).

Furthermore, the use of multiple measurements methods makes it difficult to understand results from different investigations (203): for example, some studies report LPS levels in weight per water volume (pg/mL), while others report endotoxin units per unit volume (EU/mL), which represents LPS activity (Massier et al. 2021). Given that host-derived protein binding and clearance actively regulate LPS activity, it's doubtful that LPS measurement after pretreatment to overcome limited recovery is representative of in vivo settings. This is corroborated by the fact that endotoxemia and Gram-negative bacteremia have poor concordance, and endotoxemia is identified in less than half of patients with Gram-negative sepsis (Chakaroun et al. 2020).

LBP level has been suggested as a good clinical marker of effective metabolic endotoxemia because LPS has a short half-life and measurement in biologic fluids has several limitations. This, combined with the relatively slow rise of LBP, which could serve to monitor the interaction between LPS and innate immune cells, has led to the suggestion that LBP level is a good clinical marker of effective metabolic endotoxemia (Schumann 2011).

Bacteria and Bacterial DNA

As with LPS, there is evidence for the presence of bacteria in blood, even in healthy subjects (100). Bacteria itself or bacterial DNA have been considered as possible contributors to metabolic disease. The evidence is not only for the presence of bacteria or bacterial DNA in blood but also for the association of bacterial load and bacterial composition with metabolic disorders. The amplification of bacterial DNA followed by sequencing allows the identification of microbial composition to the species level (Massier et al. 2021). It was found that numerous bacterial phyla are present in blood, with *Proteobacteria* and *Firmicutes* being the most prevalent (Massier et al. 2020). The first study linking bacterial presence in the blood with metabolic disorder, as part of a longitudinal study aimed at understanding the complicated pathophysiology of the metabolic syndrome was published in 2011 (Amar et al. 2011a). The amounts of the 16S rRNA gene were measured in 3280 people at the start of the study and after 9 years, Subjects who developed T2DM had significantly higher levels of bacterial DNA at baselines. Furthermore, when compared to healthy controls, the detection rate for bacterial DNA was considerably greater in T2DM patients (Sato et al. 2014; Abd Elaaty et al. 2019).

Other Bacterial Translocation Markers

Endotoxin core antibodies (EndoCAb) were also used as biomarkers and found to be superior to endotoxin; however, only IgM antibodies were substantially different between lean participants, obese patients, and T2DM patients (Hawkesworth et al. 2013). When compared to their lean counterparts, EndoCab IgM levels were significantly lower in obese and obese diabetic women. This could be explained by the deterioration of the IgM-LPS complex, which is responsible for constantly neutralizing endotoxin leaks from the gut. Given the lack of a definite cut-off number for this procedure as well as the lack of understanding of IgM and IgG kinetics, it is not recommended to be used as part of standard testing (Chakaroun et al. 2020).

PG is found in both Gram-positive and Gram-negative bacteria, accounting for approximately 70% and 20% of bacterial cell walls, respectively. PG binds to TLR2 and induces inflammatory response. As a quantitative assay for PG in plasma, the silkworm larvae plasma (SLP) test was developed (Kobayashi et al. 2000).

On motile bacteria, flagellin is a monomeric subunit of flagella. Flagellin binds to basolateral TLR5 on gut epithelial cells, causing inflammatory cytokines and chemokines to be secreted (Gewirtz et al. 2001). It was found that the expression of bacterial flagellin-recognizing TLR5 in adipose tissue was linked to liver fat content and insulin sensitivity in healthy women (Munukka et al. 2016). A wide range of Gram-negative flagellin is detected by ELISA.

So far, only a few studies have looked at peptidoglycan, flagellin, and their antibodies as serum bacterial translocation markers in metabolic diseases. More research in a variety of therapeutic settings is required.

Lipoteichoic acid (LTA) is a Gram-positive bacterium cell wall component. Despite the fact that LTA is the functional equivalent of Gram-negative bacteria endotoxin, it triggered differential cytokine/chemokine release via effects distal to NF-B/AP-1 activation via the TLR2 cluster (Finney et al. 2012). Unlike endotoxin, the level of LTA in the blood has not been linked to clinical inflammation.

Summary of bacterial translocation markers studied in T2DM are mentioned in Table 1.

Applications to Other Diseases/Conditions

The presence of LPS and other bacterial translocation products in systemic circulation has been linked to a variety of metabolic and non-metabolic disorders, including but not limited to: Parkinson's disease (Hasegawa et al. 2015), multiple sclerosis (Camara-Lemarroy et al. 2020), arthritis (Fotis et al. 2017), and several autoimmune diseases (Fasano 2012). The immune-escape of cancer cells is assumed to be based on a chronic inflammatory state as well as endotoxin tolerance leading to a compensatory hypoinflammatory state (Wirthgen and Hoeflich 2015).

Bacterial translocation was found to be an early event in SARS-CoV-2 infection, and it may be linked to intestinal damage from tissue infection, systemic inflammation, and IL-6-mediated vascular damage. Disruption of the intestinal barrier in

Table 1 Bacterial translocation markers studied in type 2 diabetes mellitus

Bacterial translocation marker	Study subjects	Aim of the study	Method of testing	Result	Reference (author, year)
LPS (endotoxin)	25 T2DM 25 controls	To study circulating LPS in T2DM subjects.	Chromogenic quantitative limulus amebocyte lysate (LAL) test (Cambrex).	LPS increased TLR-2 expression twofold ($P < 0.05$) and significant increase in TNF-alpha and IL-6 secretion by adipocytes	Creely et al. (2007)
LPS (endotoxin)	346 T2DM 67 controls	To investigate the relationship between endotoxin and various metabolic parameters of diabetic patients	Chromogenic kinetic LAL assay (BioWhitaker, Walkersville MD)	Endotoxin levels were elevated in all of the treated diabetic subgroups compared to controls	Al-Attas et al. (2009)
LPS (Endotoxin)	15 obese 12 impaired glucose tolerance, 18 T2DM 9 healthy controls	To evaluate the changes in circulating endotoxin after a high–saturated fat meal and to determine whether these effects depend on metabolic disease state.	Commercially available QCL-1000 LAL End Point Assay (Lonza, Allendale, NJ)	Exposure to a high-fat meal increased circulating endotoxin irrespective of metabolic state (1 h after a meal).	Harte et al. (2012)
LPS (Endotoxin) EndoCAB	29 T2DM 31 lean 33 obese	To investigate metabolic endotoxemia in Gambian women.	Endpoint Chromogenic LAL Test for Gram-negative bacterial endotoxin (LAL QCL-1000, Basal, Switzerland) Endotoxin-core IgM and IgG antibodies (EndoCAb) ELISA	LPS levels were highest in the obese-diabetic group compared with the other two groups. IgM EndoCAb was significantly reduced in the obese and obese diabetic women compared with lean women.	Hawkesworth et al. (2013)
LPS (Endotoxin)	45 T2DM 45 controls	To investigate the serum levels of LPS, zonulin, TNF-alpha and Interleukin-6 in patients	Quantitative LAL test for Gram-negative bacterial endotoxin [Lonza, USA; Cat No. QCL-1000].	LPS levels, LPS activity, and zonulin were significantly higher in patients with T2DM and showed positive	Jayashree et al. (2014)

		with T2DM compared to controls		correlation with inflammatory markers and poor glycemic/ lipid control.	
LPS (Endotoxin) LBP	80 nonobese postmenopausal women 80 diabetic nonobese postmenopausal women	To evaluate postprandial endotoxemia in postmenopausal women non-obese and diabetic patients.	Chromogenic LAL assay (QCL-1000, Lonza Group Ltd.). Plasma LBP levels were determined by a sandwich ELISA	Both study groups showed a significant increase in LPS levels and circulating LBP in plasma after the meal intake.	Zaman and Zaman (2015)
LBP	95 T2DM 40 glucose intolerance 87 controls	To study the association of circulating LBP with insulin resistance.	Serum LBP levels were measured by human LBP ELISA kit (HyCult biotechnology b.v.).	Circulating LBP was significantly increased in T2DM subjects and dramatically increased in subjects with morbid obesity.	Moreno-Navarrete et al. (2012)
LBP	44 obese 43 controls	To study the association between circulating plasma LBP levels and obesity-related biomarkers in the pediatric population	Plasma LBP were measured using commer cially available enzymelinked immunosorbent assay (ELISA) kits (Duoset, R&D Systems, Minneapolis, MN, USA)	Circulating plasma LBP levels were significantly increased in obese compared with those with normal-weight subjects. LBP levels were significantly and positively associated with insulin resistance.	Kim et al. (2016)
LBP	580 African ancestry men	To study the association between LBP and obesity and metabolic disorders.	Serum LBP was measured using a Human LBP ELISA kit (Cell Sciences, Canton, MA)	LBP was significantly associated with BMI, waist circumference, whole- body and trunk fat, skeletal muscle density, fasting serum insulin, and HOMA- insulin resistance.	Tilves et al. (2016)

(continued)

Table 1 (continued)

Bacterial translocation marker	Study subjects	Aim of the study	Method of testing	Result	Reference (author, year)
Live bacteria	50 T2DM 50 controls	To investigate the level of live gut bacteria in the systemic circulation of Japanese patients with type 2 diabetes.	16S rRNA-targeted quantitative RT–PCR using the Yakult Intestinal Flora-SCAN.	Gut bacteria were detected in blood at a significantly higher rate in diabetic patients than in control subjects and most of these bacteria were Gram-positive.	Sato et al. (2014)
Bacterial DNA	58 obese	To evaluate the incidence of bacterial translocation and the systemic inflammatory response in morbidly obese patients, and how a dietary intervention followed by bariatric surgery may impact these processes.	Broad-range PCR and of prokaryote 16SrRNA gene	Bacterial DNA translocation holds increased insulin resistance and systemic inflammatory levels in morbidly obese patients despite significant weight loss	Ortiz et al. (2014)
Bacterial DNA LBP	30 T2DM 30 controls	To evaluate the potential impact of LBP and DNA translocation on glycemic control and progression to diabetic kidney disease in T2DM patients.	Detection of bacterial DNA in blood was performed using PCR targeting 16SrRNA gene. Plasma level of LBP was determined by a commercially available double antibody sandwich ELISA (Assay kit Co., Ltd, USA)	Plasma levels of LBP were significantly elevated in T2DM than in controls. LBP level was significantly and positively correlated with fasting glucose level, albumin-creatinine ratio and negatively correlated with glomerular filtration rate.	Abd Elaaty et al. (2019)

DNA: Deoxyribonucleic acid, ELISA: enzyme linked immunosorbent assay, LBP: lipopolysaccharide binding protein, LPS: lipopolysaccharide, RT-PCR: Reverse transcriptase-Polymerase chain reaction, T2DM: Type 2 diabetes mellitus, 16SrRNA: 16S ribosomal ribonucleic acid

COVID-19 patients results in the systemic release of PAMPs, such as LPS, and consequently TLR activation, which then initiates vicious cycles of systemic inflammation and tissue damage. Thus, bacterial translocation could serve a synergistic role in the cytokine release syndrome in COVID-19 (Gao et al. 2020).

These studies indicate a causal link between gut-derived LPS or bacterial translocation products and disease progression, and they emphasize the need of maintaining gut barrier function as well as focused regulation as a novel therapeutic option.

Mini-Dictionary of Terms

- Gut Microbiome vs gut microbiota: the two terms are used interchangeably. Microbiota refer to all types of microorganisms (bacteria, fungi, archae, viruses) that inhabit the gastrointestinal tract (GIT). Microbiome refer to the microorganisms and their genes that inhabit the GIT.
- Gut dysbiosis: imbalance in the gut microbiome composition that is mostly associated with diseases.
- Bacterial translocation: the passage of microorganisms from the GIT to the circulation.
- Metabolic endotoxemia: high level of endotoxins (LPS) in blood which occurs due to passage of bacterial LPS from the gut to the circulation.

Key Facts

- Type 2 Diabetes mellitus (T2DM) is a metabolic disorder, multifactorial in origin.
- T2DM is characterized by insulin resistance and a low-grade inflammation.
- Metabolic endotoxemia triggers insulin resistance.
- Lifestyle changes including diet control can help prevent or delay the onset of T2DM.
- Better understanding of the relationship between gut microbiome and T2DM is still needed.

Summary Points

- Type 2 Diabetes mellitus (T2DM) is a metabolic disorder, multifactorial in origin.
- Altered gut microbiome in T2DM has led to the emergence of the concept of "leaky gut" and metabolic endotoxemia.
- Increased intestinal permeability allows the translocation of bacteria and their products through the gut barrier.
- Translocated bacterial products stimulate a systemic inflammatory response affecting many organs and increasing insulin resistance that help diabetes progression.

- Better understanding of the mechanism of leaky gut and bacterial translocation in diabetes will help scientists to develop novel diagnostic and therapeutic approaches.

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Serum Leptin as a Biomarker in Diabetes

46

Links with Acromegaly

Hind Shakir Ahmed

Contents

Diabetes Mellitus 949
Complications of Diabetes Mellitus 949
Insulin Resistance and β-Cell Function in Type 2 Diabetes Mellitus 950
Diabetes Mellitus and Acromegaly 951
Acromegaly Comorbidities 951
Metabolic Complications 951
Cardiovascular Disease 952
Insulin Resistance and Leptin in Type 2 Diabetes Mellitus 954
Leptin Resistance in Type 2 Diabetes Mellitus 956
Leptin in Acromegaly 956
Medical Therapy 957
Applications to Prognosis 957
Applications to Other Diseases or Conditions 958
Mini-Dictionary of Terms 958
Key Facts of Hyperinsulinemia 958
Key Facts of Human Leptin 959
Key Facts of Acromegaly 959
Summary Points 959
References 960

Abstract

Acromegaly is a rare disease caused by an overproduction of growth hormone and revealed by progressive clinical features. Due to the abundance of growth hormone receptors in the body, it is a systemic disease that leads to many complications and concomitant diseases. An obvious medical concern in the

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context of acromegaly is diabetes. It can be the result of excess growth hormone and its mediators, but it can also result from the treatment of acromegaly.

This chapter reveals the pathophysiological role of leptin in glucose and lipid metabolism in diabetic patients, especially those with acromegaly. In addition, there is a section highlighting the effect of acromegaly treatment on glucose metabolism, including approved drugs as well as those currently being explored. It also reviews the observations of the use of antidiabetic drugs in acromegaly patients.

Glucose homeostasis is an important feature of the comorbidity of acromegaly and has further care. Although several trials have tested glucose homeostasis in acromegaly, more basic, translational, and clinical investigations are still needed to better consider the underlying mechanisms and how to better report them.

Keywords

Diabetes mellitus · Type 2 diabetes mellitus · Insulin resistance · Homeostatic model assessment · Pancreatic β-cell function · Growth hormone · Insulin-like growth factor-1 · Acromegaly · Leptin · Leptin resistance

Abbreviations

CV	Cardiovascular
CVD	Cardiovascular disease
DM	Diabetes mellitus
ERK	Extracellular signal-regulated protein kinase
FA	Fatty acid
FBG	Fasting blood glucose
FFAs	Free fatty acids
GH	Growth hormone
gp	Glycoprotein
HDL	High-density lipoprotein
HOMA%B	Homeostatic model assessment for β-cell function
HOMA%S	Homeostatic model assessment for insulin sensitivity
HOMA	Homeostatic model assessment
HTN	Hypertension
IFG	Impaired fasting glucose
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IR	Insulin resistance
IRS	Insulin receptor substrate
IS	Insulin sensitivity
JAK	Janus family of tyrosine kinases
LDL	Low-density lipoprotein
MAP	Mitogen-activated protein
PKC	Protein kinase C

STAT	Signal transducers and activators of transcription
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TNF-α	Tumor necrosis factor-α
WHO	World Health Organization

Diabetes Mellitus

Diabetes mellitus (DM) is one of the most common metabolic disorders manifested by hyperglycemia. It occurs due to total or partial insulin deficiency and/or insulin resistance (IR). The World Health Organization reports that DM bothers about 422 million adults in the world. In 2019, diabetes was the seventh cause of death and the eighth cause of disability (WHO 2019).

Diabetes is a public health threat that afflicts millions of people in the world of all ages, gender, race, and ethnicity (Wondafrash et al. 2020). The burden of diabetes is total worldwide, and about 80% of DM deaths occur predominantly in low- and middle-income countries (Afroz et al. 2019). Based on the pathogenesis of hyperglycemia, most diabetic patients are either type 1 (insulin-dependent T1DM) or type 2 (non-insulin-dependent T2DM). Also, T2DM has been most frequently documented (90–95% of patients) (Chen et al. 2017).

The pathologic hallmarks of T2DM are primarily unusual insulin secretion and higher insulin resistance (IR). There is also a decline in the functional pancreatic β-cell mass over time upon the progress of the disease situation. Individuals with impaired fasting glucose (IFG) were revealed a decrease of 40% in relative β-cell volume, proposing that loss of β-cell mass was surviving at early stages of T2DM. Moreover, it was documented that β-cell mass was already decreased by 50% at the time of diagnosis of T2DM and that it continued to deterioration through the course of T2DM and the diminished β-cell mass was not due to decreased formation of islets or regeneration but was caused by greater rates of apoptosis in islets (Zaccardi et al. 2016). Deterioration in cell function in T2DM leads to progressive deterioration of glycemic control and increases the need for insulin. Moreover, T2DM is primarily associated with obesity, which results in decreased glucose-mediated insulin uptake due to impaired intracellular insulin signaling. There is also decreased secretion of glucagon-like peptide 1, which is secreted after eating and increases insulin secretion in T2DM patients (Murakami et al. 2017).

Complications of Diabetes Mellitus

If left untreated, diabetes is associated with bothersome short-term complications (diabetic ketoacidosis and hyperglycemic state) and long-term complications (retinopathy, nephropathy, neuropathy, and lower claudication amputation). Moreover, various cardiovascular (CV) and endocrine defects are associated with diabetes, which may disturb patients' quality of life (Wang and Lo 2018).

Insulin Resistance and β-Cell Function in Type 2 Diabetes Mellitus

Insulin resistance is a condition in which peripheral tissues, i.e., the liver, skeletal muscle, and adipose tissue, do not respond to normal insulin levels. As a result, pancreatic cells secrete greater amounts of insulin as a compensatory mechanism. This constitutes a significant biological and energy burden on cells. When β-cells fail to produce elevated insulin in response to greater metabolic demand, T2DM results emerge (Newsholme et al. 2016).

Hyperinsulinemic-euglycemic clamp technique is deliberated to be the best method to assess even lesser variants in insulin sensitivity (IS), though it is burdensome to achieve, labor-intensive, and expensive (DeFronzo et al. 1979).

The homeostatic model assessment (HOMA) test was presented as a simple method that mathematically uses fasting blood glucose (FBG) and insulin levels and provides an assessment of IS score (HOMA%S) and cell function (HOMA%B). Hence, HOMA-IR is the reciprocal of HOMA%S (Matthews et al. 1985). The simplified HOMA model (HOMA2) in addition to the steady-state glucose and insulin ratio also reflects variables in hepatic and peripheral glucose resistance, i.e., lessening in suppression of hepatic glucose production, increase in insulin secretion curve of plasma glucose levels greater than 10 mmol/L, and influences of proinsulin turnover (Levy et al. 1998).

The IR modifier was also calculated using the HOMA2 calculator program. The HOMA2 calculator was downloaded from the University of Oxford (http://www.dtu.ox.ac.uk/). Subjects with a HOMA2-IR greater than 1.8 were defined as the IR group, while subjects with a HOMA2-IR less than or equal to 1.8 were defined as the IS group. Computer models were used to produce a modular chart by which mathematical transformations of FBG and insulin statistics from individuals identify distinct groups of HOMA%S and HOMA%B from steady-state states (Kalaichelvi and Somasundram 2016).

In addition, these models using insulin have some constraints. First, insulin secretion is pulsatile, which limits the usage of a single sample for insulin assessment. Alternatively, an average of at least three samples taken at 5-min intervals was used to calculate HOMA to obtain more consistent results. Moreover, careful phlebotomy is necessary to avoid hemolysis as far as the hemolytic effects of insulinolysis can occur. Moreover, approximately 50% of the insulin secreted by β-cells is removed by the liver, resulting in a large inter-assay insulin variant (Wallace et al. 2004).

In China, researchers found HOMA modified with C-peptide to exchange insulin in HOMA to evaluate islet cell function and IR in healthy individuals and diabetic states. This method appears to be more suitable as a range of insulin secretion since the secreted C-peptide is not extracted by the liver and other organs and also, the serum C-peptide half-life is longer than that of insulin (10-30 min vs. 4 min). Hence, assessment of C-peptide is a more reliable feature for beta-cell insulin secretion compared to an assessment of insulin (Li et al. 2004).

The pathways to β-cell demise and dysfunction are less well differentiated in T2DM, but β-cell insulin deficiency, often in IR, appears to be the mutual

denominator. In addition, T2DM is associated with defects in insulin secretion due to metabolic stress or inflammation in some individuals, including genetic influences. Future classification schemes for DM will provide a prospective focus on the pathophysiology of the primary cell defect. It has also revealed that it takes 5–10 years before the true symptoms of DM appear due to cell dysfunction and IR (Chung et al. 2020).

Diabetes Mellitus and Acromegaly

The leading physiological action of growth hormone (GH) is the regulation of postpartum growth and lipolysis. These actions are highly dependent on the nutritional status, which is involved in the metabolism of GH (Facey et al. 2017). The GH secretion is improved by fasting, GH-releasing hormone (GHRH), stress, physical activity and hypoglycemia, and inhibited by insulin, glucose free fatty acids (FFAs), insuline-like growth factor-1 (IGF-1) and somatostatin. Excess GH in acromegaly, with few exceptions, results in a benign tumor of somatic pituitary cells and leads to chronically higher GH levels, which do not respond to the usual physiological inhibition of the feedback (Hawkes and Grimberg 2015).

The IGF-1 is a single-chain polypeptide containing 50% of the amino acid sequence homologous to insulin, so sharp increases in IGF-1 levels result in insulin-like properties in glucose transport and circulating glucose levels, as well as in insulin receptor deficiency. However, circulating IGF-1 does not cause hypoglycemia, as more than 90% binds to specific binding proteins (Frara et al. 2016).

Acromegaly is a rare disease interrelated with higher morbidity due to secondary systemic complications involving cerebral, CV, respiratory, and osteoarticular systems, as well as endocrine, metabolic, and neoplastic alterations (Gadelha et al. 2019).

Acromegaly Comorbidities

Metabolic Complications

Acromegaly patients have metabolic disorders that disturb both glycemic and lipid metabolisms and are usually due to excess GH. Most patients with acromegaly have IR with poor IS, and improved gluconeogenesis in the liver and kidneys, which leads to glycemic irregularity. In the fasting state, GH is the main anabolic hormone that resists insulin, and the excess leads to the continuous stimulation of lipolysis and fat oxidation (Bolfi et al. 2018). Growth hormone inhibits lipoprotein lipase activity in adipose tissues, leading to an increase in the efflux of FFAs to the liver, which in turn favors IR, elevates the synthesis of triacylglycerol (TAG), and decreases high-density lipoprotein (HDL) levels and percentage of body fat (BF%). This continued lipolysis leads to acromegaly's distinctive metabolic alterations in which IR can be seen along with decreased body fat. Furthermore, decreased glucose uptake in

Table 1 Metabolic effects of GH and IGF-1

Metabolic pathway	GH	IGF-I
Glucose uptake	↓	↑
Insulin secretion	↑	↓
Insulin sensitivity	↓	↑
Lipolysis	↑	–
Protein synthesis	↑	–
Protein breakdown	↑	–

↑ stimulating, ↓ inhibiting

adipose tissue and muscle with lesser expression of glucose transporter-1 and glucose transporter-4 is also detected in acromegaly (Vila et al. 2019) (Table 1). On the contrary, IGF-1 has contradictory actions comparable to GH under physiological situations. It stimulates FFA uptake into adipose and liver tissues, leading to decreased FFA, in addition to improved glucose uptake and IS mainly on skeletal muscles. Nevertheless, in acromegaly, IR dominates and IGF-1's possibly constructive effects are countered. This leads to higher occurrence of glucose and lipid irregularities in acromegalic patients as comparable to normal subjects (Vilar et al. 2007).

In newly diagnosed acromegalic patients, about 50% of cases lead to glucose impairment manifested by altered FBG, impaired glucose tolerance (IGT), or DM. Regarding DM alone, it is existent in nearly 30% of cases, but has been recognized in up to 56% of cases, dependent on the existence of other risk features. Severity of glucose defects is associated with older age, greater body mass index (BMI), and positive family history of DM, in addition to GH and IGF-1 levels (Alexopoulou et al. 2014).

Furthermore, hyperlipidemia is also existent in up to 50% of acromegalic patients and is primarily manifested by hypertriglyceridemia and decreased HDL levels. Though low-density lipoprotein (LDL) levels have been higher or similar to normal individuals, higher concentrations of oxidized LDL have been documented in these cases. Therefore, IR is the leading metabolic irregularity seen in acromegalic patients (Boero et al. 2010).

Impaired glucose uptake correlates with activation associated with lipolysis, in which administration of the anti-lipolytic agent acipimox abrogates the actions of GH on IS (Nielsen et al. 2001) (Table 2).

Cardiovascular Disease

Cardiovascular disease (CVD) is one of the most predominant comorbidities in acromegalic patients, with arterial hypertension (HTN) being the greatest public illness, with predominance fluctuating 18–60% and being existent since early times. It is categorized by higher diastolic blood pressure and higher predominance of non-dippers (Ramos-Levi and Marazuela 2019). In fact, CVD appears to lead to

Table 2 Effects of GH/IGF-1 hypersecretion in metabolic process

Stimulus factor	Healthy	Acromegaly
GH levels	Normal	↑↑
IGF-I levels	Normal	↑↑
Negative feedback of IGF-I on GH secretion	Normal	↓ GH
β-Cell function	↑	↓
Glucose uptake	Normal	↓
Insulin levels	Normal	↑
Glycogenolysis	Normal	↑
Gluconeogenesis	↑	↑
Lipolysis	↑ During fasting	↑↑

↑ stimulating, ↓ inhibiting

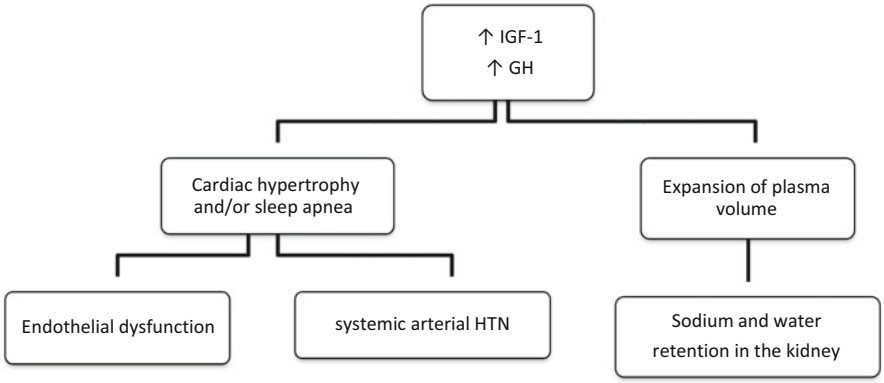

Fig. 1 The effects of GH/IGF-1 increases

death in 23–50% of cases dependent on patient's age, or years in which the experimental evidence were documented. It is identified that augmented levels of GH/IGF-1 ratio due to sodium-retaining influence initiate an elevation in extracellular volume which leads to HTN, in addition to determining morpho-functional heart changes such as diastolic dysfunction, hypertrophy, and up to systolic dysfunction in the end stage (Pivonello et al. 2017). Other illnesses, as cardiac hypertrophy and sleep apnea, may lead to the elevation in blood pressure (Fig. 1). At long-term acromegaly, there are minor modifications in the vascular system and cardiac alteration that also help aggravate arterial HTN. Subsequently, chronic IR and fatty acid-induced lipotoxicity worsen β-cell function ultimately leading to DM (Ramos-Levi and Marazuela 2017).

The incidence of DM in acromegalic patients is 20–35% at early diagnosis. The incidence of DM correlates with disease control in acromegaly, and IGF-I levels are greater in diabetic patients than in patients with IGT or normal glucose metabolism (Gonzalez et al. 2018).

Insulin Resistance and Leptin in Type 2 Diabetes Mellitus

Another mechanism of GH-induced IR is via the GH-dependent increase in plasma FFAs. The inhibition of glucose oxidation by fatty acids (FAs) is also called the Randle cycle effect. The formation of TAG from FA results in the accumulation of diacylglycerol and ceramide. These intermediate reesterification compounds result in protein kinase C (PKC) isoforms, which regulate insulin signaling through various mechanisms. Growth hormone replacement therapy is related with inhibition of glucose metabolism as a consequence of the lipolytic effect of GH (Bramnert et al. 2003). Leptin is a polypeptide hormone consisting of 167 amino acids with an N-terminal secretory signaling sequence of 21 amino acids. It is a hormone secreted by adipocytes and has been found to control food intake. Leptin assists in the homeostasis of dietary behavior through central neuroendocrine mechanisms (Friedman 2019). Leptin is encoded by the LEP gene on chromosome 7q31.3 in humans (ob gene), and its main site of action is the brain, where it acts primarily on the hypothalamus. In addition to its main activities in the brain, it also acts directly on immune cells in the periphery. It is structurally similar to cytokines and includes an intra-chain disulfide bond of functional importance. This circulating leptin positively correlates with protein concentrations and leptin mRNA in adipose tissue. The hormone acts as a regulator of energy expenditure and neuroendocrine function, and has since provided key insights into obesity (Abella et al. 2017). Its level is proportional to fat mass, and although it is mainly produced by fat cells, it is also produced by cardiac muscle cells, vascular smooth muscle cells, and the placenta in pregnant women. The functional leptin receptor is located in the hypothalamus where it increases energy expenditure and decreases appetite. These receptors have also been identified in other organs such as the heart, pancreas, liver, kidneys, blood vessels in the brain, and the myometrium (Ghantous et al. 2015).

In obesity, pancreatic cells are stimulated in numbers, and insulin is excessively increased in parallel with weight gain to recompense for higher concentrations of blood glucose and FFA (Corkey 2012). Under typical situations, leptin acts directly on leptin receptors expressed in pancreatic cells to inhibit insulin production while promoting glucose disposal in skeletal muscle and oxidative stress. Hyperinsulinemia detected in leptin-deficient (ob/ob) mice, leptin receptor-deficient (db/db) mice, and leptin-resistant humans is further indication of the reserve of leptin interaction with insulin secretion. Additionally, hyperleptinemia in obesity can lead to leptin resistance in β-cells, leading to overproduction of insulin by β-cells (Farooqi et al. 2002). Several experimental signals showed that leptin- and ObR-related genes and leptin-related genes were identified in human tissues from various brain tumors. Therefore, additional clinical evidence is needed to clarify the particular characteristics and biological role of the leptin/ObR axis for many individuals with brain tumors (Knerr et al. 2001).

In fact, insulinoma patients have higher leptin levels. Moreover, leptin monotherapy has been revealed to control a variety of complications of diabetes in rats which include hyperketemia, hyperglycemia, hyperglucagonemia, and polyuria due to lack of insulin (Denroche et al. 2011), although leptin therapy

has not been effective in cases of T2DM associated with obesity. Therefore, these results revealed the sequence of sequential events; fat accumulation during obesity leads to a rise in leptin and FFA levels (hyperlipidemia), which leads to the progress of leptin resistance followed by a rise in insulin secretion. Thus, these actions can stimulate tissue-specific infrared radiation (Febbraio 2007). Furthermore, it is recommended that leptin have pro-inflammatory property due to its cytokine-like structure and degree of sequence homology between leptin receptor (LRb) and glycoprotein 130 (gp130); activation of both receptors triggers the Janus family of tyrosine kinases/signal transducers, activators of transcription (JAK/STAT), and extracellular protein kinase (ERK) signaling pathways. The JAK/STAT pathway involves signaling from cytokine receptors to the nucleus. Hence, JAKs are triggered by activation of the cytokine receptor. Stimulation of JAKs leads to the activity of the STAT transcription factor. Several potential roles for the diverse domains of JAK proteins have been proposed. Leptin not only drives the production of pro-inflammatory mediators such as interleukin (IL)-2 and interferon-gamma (IFN-γ) but also inhibits the production of the anti-inflammatory cytokine IL-4 by T cells or mononuclear cells. The collaboration between leptin and inflammation is bidirectional as leptin is overexpressed in response to pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α) and IL-1] and endotoxin (lipopolysaccharide), which prolongs the chronic inflammatory state in obese subjects. All of these actions increasingly lead to cell failure, and thus are due to overt T2DM (Scarpace and Zhang 2007).

Obesity is associated with high concentrations of leptin. Chronic overexpression of leptin downregulates leptin receptors, thereby reducing signaling and impairing sensitivity to exogenous leptin, suggesting that obese individuals are resistant to the adipocyte hormone (Carlyle et al. 2005). Besides its effect on appetite and metabolism, leptin works in the hypothalamus to raise blood pressure by activating the sympathetic nervous system. High levels of circulating leptin have been described to explain much of the increase in renal sympathetic tone detected in obese individuals. Leptin also disturbs vascular structure by promoting angiogenesis and atherosclerosis (De Luis et al. 2009).

Mutation of the obesity-causing gene leads to increased food intake, increased insulin levels, and observed obesity in patients with T2DM. The mutated gene causes stable or no leptin production. However, such mutations are very unfavorable in humans (Myers et al. 2010). These effects can be reversed by administering leptin. This adipocyte-specific protein provides the first links to the body's system that controls body weight. Although leptin is usually elevated with obesity, it has been shown that at every BMI score, there is asymmetry in the level of leptin in the blood. This indicates that there are differences in the rate of its excretion of fat. Assuming that the presence of leptin diminishes food intake and reduces body weight, increased leptin concentrations among obese subjects would be considered leptin-resistant. In these conditions, humans lack sensitivity to appetite to reduce the influences of leptin. The effects of leptin resistance are subject to change. It is hypothesized that decreased leptin detection in melanocortin circuits affects leptin-resistant diseases (Lin et al. 2000).

Leptin Resistance in Type 2 Diabetes Mellitus

High concentrations of circulating leptin, characteristic of obesity, combined with poor response to the compulsive properties of leptin are indicative of leptin resistance (Farcas et al. 2018). Leptin resistance due to the high-fat diet leads to defect in the hypothalamus, which considerably reduces the capability of peripheral leptin to activate hypothalamic signaling. Resistance also occurs due to a defect in intracellular signaling in leptin-responsive hypothalamic neurons (German et al. 2010). Leptin has also been related with the disease atherosclerosis. The thickening of the intima and tunica media is supposed to be a marker of the initial stage of atherosclerosis, before symptoms get up. Researchers indicated that serum leptin levels are independently and positively associated with the intima-media thickness of the common carotid artery. This proposes that elevation in leptin level is a risk factor for the progress of atherosclerosis (Aragones et al. 2016). Given that obesity can be a consequence of leptin resistance, it is likely that leptin resistance is involved in disease development. Like leptin resistance, leptin deficiency is important in causing severe IR in uncontrolled insulin deficiency DM. However, it may be indicated that leptin signaling can be restored in neurons by overexpression of endogenous peptides and/or suppression of native peptides. Dietary composites, i.e., taurine, caffeine, and cholesterol, are able to return leptin signaling in neurons using the expression or suppression of these peptides. It has also been concluded that some vitamins such as A and D enhance the transport of leptin across the blood-brain barrier (Casanueva and Dieguez 1998).

Furthermore, a significant decrease has been found in serum leptin levels after metformin and empagliflozin administration in rat models, which demonstrated that empagliflozin caused reduced serum leptin levels (Mironova and Hanjieva-Darlenska 2020).

Leptin in Acromegaly

Growth hormone is known to be associated with changes in body composition. Children and adults with growth hormone deficiency (GHD) had abnormal body composition, with increased fat mass and lower fat mass along with higher concentrations of leptin, compared with age, BMI, and sex-matched healthy individuals, while acromegaly improved lean body mass. Lowered BF% indicates that leptin is a marker of body composition and not fat mass alone. Treatment with recombinant human growth hormone in adults with GHD, as well as children with GHD caused by marked decreases in fat mass and leptin levels, lowers serum leptin concentrations in active acromegaly, and thus may be explained, at least in part, by increased lean body mass and lowered BF (Flier 1997). Hyperactivity of the GH/IGF-I axis in acromegaly patients results in IR in the liver and extremities. These patients show hyperinsulinemia and increased glucose turnover in the basal and postabsorptive states. The increase in GH among normal and insulin-deficient men increases the body's production of bioavailable FA and ketones by stimulating lipolysis, and these

effects may be due to impaired IS. Thus, there is a possibility that the rate of glucose uptake, which is the maximum rate of TAG storage in fat cells, rather than insulin and glucose per se, may indicate hemostasis of leptin gene expression (Rosenbaum et al. 1996), a condition in which insulin action and/or secretion is impaired in acromegaly and thus may contribute to a further decrease in leptin levels. Another example of these findings is that leptin travels with fluctuations in body composition. Since chronic overactivity of the GH and IGF-1 axis disturbs both the liver and peripheral tissues, it may be necessary to assess leptin with liver-derived IGF-1 (Wawrzkiewicz-Jałowiecka et al. 2021). It has been postulated that liver-derived IGF-1 is not required for postnatal body development in mice with complete inactivation of the IGF-1 gene in hepatocytes. Blood leptin levels can be affected by the function of the gonads. Estrogen plays a regulatory role in leptin secretion in women by controlling the synthesis of leptin coding transcripts and the expression of specific leptin receptors (Jenks et al. 2017), but E2 exerts no effect on adipocytes in men. A conflicting result is used by androgens. Both testosterone and dihydrotestosterone reduce leptin gene expression and secretion from human adipocytes (Casabiell et al. 1998).

Medical Therapy

Although three modalities of treatment (surgery, medicinal treatment, and radiotherapy) are obtainable and new drugs were appropriate in the last periods, there are still some patients that retain disease activity despite treatment. Hence, there is a requirement for new therapies for acromegalic patients, and also new drugs are presently under study. These treatments involve somatostatin receptor ligands (i.e., octreotide, lanreotide, and pasireotide), dopamine agonists, and the GH receptor antagonist pegvisomant (Lamberts and Hofland 2019).

Applications to Prognosis

The homeostatic model assessment (HOMA) test was presented as a simple method that mathematically uses fasting blood glucose (FBG) and insulin levels and provides an assessment of IS score (HOMA%S) and cell function (HOMA%B). Hence, HOMA-IR is the reciprocal of HOMA%S (Matthews et al. 1985). The simplified HOMA model (HOMA2) in addition to the steady-state glucose and insulin ratio also reflects variables in hepatic and peripheral glucose resistance, i.e., lessening in suppression of hepatic glucose production, increase in insulin secretion curve of plasma glucose levels greater than 10 mmol/L, and influences of proinsulin turnover (fibrillary) (et al. 1998). The IR modifier was also calculated using the HOMA2 calculator program. The HOMA2 calculator was downloaded from the University of Oxford (http://www.dtu.ox.ac.uk/). Subjects with a HOMA2-IR greater than 1.8 were defined as the IR group, while subjects with a HOMA2-IR less than or equal to 1.8 were defined as the IS group. Computer models were used to produce a

modular chart by which mathematical transformations of FBG and insulin statistics from individuals identify distinct groups of HOMA%S and HOMA%B from steady-state states (Kalaichelvi and Somasundram 2016).

Applications to Other Diseases or Conditions

The leading physiological action of growth hormone (GH) is the regulation of postpartum growth and lipolysis. These actions are highly dependent on the nutritional status, which is involved in the metabolism of GH. Growth hormone is released from somatic pituitary cells in a pulsatile mode regulated by hormones and nutrition (Facey et al. 2017). The GH secretion is improved by fasting, GH-releasing hormone (GHRH), stress, physical activity, and hypoglycemia, and inhibited by insulin, glucose, free fatty acids (FFAs), insulin-like growth factor-1 (IGF-1), and somatostatin. Excess GH in acromegaly, with few exceptions, results in a benign tumor of somatic pituitary cells and leads to chronically higher GH levels, which do not respond to the usual physiological inhibition of the feedback (Hawkes and Grimberg 2015).

The IGF-1 is a single-chain polypeptide containing 50% of the amino acid sequence homologous to insulin, and is stimulated not only by GH but also by insulin. Sharp increases in IGF-1 levels result in insulin-like properties in glucose transport and circulating glucose levels, as well as in insulin receptor deficiency. However, circulating IGF-1 does not cause hypoglycemia, as more than 90% binds to specific binding proteins (Frara et al. 2016).

Acromegaly is a rare disease manifested by intense GH and enhanced IGF-1, which, in the vast majority of cases, is caused by GH-secreting pituitary adenoma. It is associated with higher morbidity due to secondary systemic complications involving cerebral, CV, respiratory, and osteoarticular systems, as well as endocrine, metabolic, and neoplastic alterations (Gadelha et al. 2019).

Mini-Dictionary of Terms

- DKA has been reported to occur in children and adult patients with T2DM.
- IR is a pathophysiological condition associated with DM, obesity, and MetS.
- Acromegaly is characterized by profound conflicts of carbohydrate and lipid metabolism.
- Leptin is a protein hormone that regulates food intake and energy expenditure.
- Leptin resistance is associated with hyperinsulinemia in T2DM and obese subjects.

Key Facts of Hyperinsulinemia

- DKA is an acute complication of uncontrolled DM that is complicated with higher morbidity and mortality.

- Hyperinsulinemia is described by irregularly high insulin levels in the blood, and this condition is associated with T2DM, but alone it is not theoretically a form of DM. Hyperinsulinemia is also a factor in IR, obesity, and metabolic syndrome.

Key Facts of Human Leptin

- Leptin has structural homology to TNF-α, IL-6, leukemia inhibitory factor, granulocyte colony-stimulating factor, gp130, and other cytokine family proteins, and then is measured a cytokine-like substance.
- Five isoforms of leptin receptors have been identified, namely, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re. The leptin receptor is a transmembrane protein with structural similarity to the gp130 receptor family. The extracellular domain of the leptin receptor comprises two cytokine binding regions with one region being the particular leptin binding site.
- The intracellular domain usually associates with one or two box motifs, while most gp130 receptors have three box motifs. Cytoplasmic forms of leptin receptors allow interaction with intracellular messengers, i.e., mitogen-activated protein (MAP) kinase, insulin receptor substrate (IRS-1 and IRS-2), nitric oxide, JAK, and STAT signaling.

Key Facts of Acromegaly

- Medical treatment is currently an important management choice and can even be the first treatment in acromegalic patients who will not benefit or are unsuitable for first-line neurosurgical management.

Summary Points

- Acromegaly is a systemic disease associated with increased morbidity, presenting CV, metabolic, respiratory, neoplastic, endocrine, joint, and orthopedic complications.
- Most comorbidities of patients with acromegaly can be prevented or impeded by appropriate treatment, which has revealed a change in the severity and incidence of these comorbidities.
- Growth hormone secretion is predominantly under the regulation of two hypothalamic neuroendocrine hormones: GH-releasing hormone (GHRH), which triggers growth hormone secretion, and somatostatin, which restricts GH secretion. Several other endocrine mediators also control the transcription of the GH gene, including IGF-1, which is the major suppressor of GH transcription.
- It is well known that GH stimulates glucose production in the liver, but it is uncertain whether GH positively triggers glycogenolysis or gluconeogenesis.

- The pathway for insulin stimulation of glucose transport in muscle includes activation of the insulin receptor protein, which docks IRS-1 and IRS-2 and phosphorylates these proteins on tyrosine residues.
- The state of reduced insulin action and/or secretion in acromegalic patients may lead to leptin resistance, which is related with body composition.
- Leptin in combination with dieting stimulates leptin signaling and shows therapeutic potential as a means of controlling obesity and reducing IR.
- Medical rehabilitation is suggested for acromegalic patients who do not achieve biochemical control after surgery. Primary medical therapy is reserved for those with contraindication to or who reject surgery, and may be deliberated in select patients reflected at poor risk for good results and surgical success.

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Vitreous Biomarkers: What They Are and How They May Be Used to Advance the Management of Diabetic Retinopathy

47

Ricardo Lamy and Jay M. Stewart

Contents

Introduction 965
Diabetic Retinopathy 966
Pathogenesis and Pathophysiology 966
Clinical Grading 968
Clinical Management 969
Assessing Local Molecular Biomarkers for DR 972
Ocular Liquid Biopsy 972
Sample Selection and Method of Analysis 973
Vitreous Molecular Biomarkers of DR 974
Cytokines 974
Kinin-Kallikrein System (KKS) 979
Complement System (CS) 981
MicroRNA (miRNA) 982
Metabolites 982
Applications to Prognosis and Other Diseases and Conditions 982
Key Facts of Vitreous Biomarkers in Diabetic Retinopathy (DR) 983
Mini-Dictionary of Terms 983
Summary Points 984
References 984

Abstract

Vitreous humor is a transparent gel-like tissue that fills the posterior segment of the eye, adjacent to the lens, ciliary body, and retina. It has a complex composition of water, collagen, and thousands of proteins that are released by the adjacent structures and can conversely influence the physiology of those same structures. Recent scientific advancements have allowed the investigation of numerous

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vitreous proteins as prognostic and predictive biomarkers for retinal diseases, including diabetic retinopathy (DR). DR is a common neurovascular complication of diabetes mellitus and a leading cause of blindness worldwide. Anti-VEGF therapy has revolutionized the treatment of vision-threatening stages of DR, but many patients do not respond to the treatment. Novel prognostic and predictive biomarkers in DR have potential to facilitate disease prognosis, predict the best responders to a specific treatment, and support the development of new therapies.

Keywords

Diabetes · Diabetic retinopathy · Vitreous biomarkers · Vitreous proteomics · Liquid biopsy · Molecular diagnosis · Angiogenesis · Retinal neovascularization · Proliferative retinopathy · Vascular endothelial growth factors · Drug discovery

Abbreviations

A2AP	Alpha 2-antiplasmin
ACCORD	Action to Control Cardiovascular Risk in Diabetes
AGEs	Advanced glycation end-products
AH	Aqueous humor
BK	Bradykinin
BRB	Blood-retina barrier
CCL2	Chemokine ligand 2
CXCL8	Interleukin 8
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DME	Diabetic macular edema
DR	Diabetic retinopathy
DRSS	Diabetic Retinopathy Severity Scale
EC	Endothelial cells
ECL	Electrochemiluminescent
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ET-1	Endothelin-1
ETDRS	Early Treatment Diabetic Retinopathy Study
FDA	The Food and Drug Administration
FIGF	c-Fos-induced growth factor
FXII	Coagulation factor XII
HbA1c	Glycated hemoglobin
HGF	Hepatocyte growth factor
HK	High-molecular-weight kininogen
ICAM	Intercellular adhesion molecule
IL-6	Interleukin-6
IL-8	Interleukin-8
IRMA	Intraretinal microvascular abnormality

KKS	Kinin-kallikrein system
KLKB1	Kallikrein B1
KNG	Kininogen 1
MCP-1	Monocyte chemotactic protein 1
MH	Macular hole
NF-kB	Nuclear factor kappa B
NIH	National Institutes of Health
NO	Nitric oxide
NPDR	Nonproliferative diabetic retinopathy
OCT	Optical coherence tomography
PDGF-BB	Platelet-derived growth factor BB chain
PDR	Proliferative diabetic retinopathy
PEA	Proximity extension assay
PEDF	Pigment epithelium-derived factor
PGF	Placental growth factor
PK	Plasma kallikrein
PKC	Protein kinase C
PLMN	Plasminogen
PPK	Plasma prekallikrein
PRP	Panretinal photocoagulation
RAGEs	Receptors for advanced glycation end products
RPE	Retinal pigmented epithelial cells
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
UKPDS	UK Prospective Diabetes Study
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

Introduction

Biomarker is a shortened term for biological marker and can be defined as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention (FDA-NIH Biomarker Working Group 2016). Systemic blood plasma and serum molecular biomarkers are widely used in medicine, and they have their importance in ophthalmology, but because of particular characteristics of the eye microenvironment, including the blood-retina barrier (BRB) and the small volume of the organ in proportion to the whole body, many systemic blood biomarkers do not correlate well with ocular diseases. Locally, the optical transparency of the cornea and lens allows for direct visualization of the retina and detection of several imaging biomarkers that are broadly used to diagnose, monitor, and prognosticate retinal diseases. Over the last decades, the search for local molecular biomarkers for retinal diseases has

advanced significantly with the use of novel molecular techniques capable of working with very small volumes of biospecimens. The vitreous humor milieu is rich in proteins secreted by the surrounding tissues, making it a great source of molecular biomarkers for retinal diseases, including diabetic retinopathy (DR) (Lamy et al. 2020).

To better comprehend the potential significance of vitreous biomarkers for DR, it is important to understand some current concepts about the epidemiology, pathogenesis, pathophysiology, clinical classification, and management of DR.

Diabetic Retinopathy

Diabetes mellitus (DM) is characterized by hyperglycemia, resulting from the body's inability to produce insulin and/or resistance to insulin action. Various genetic and environmental factors can contribute to DM, and patients with different forms of diabetes are at risk for developing the same chronic complications, although the incidence of complications is higher in people with DM type 1 compared with DM type 2 (Deshpande et al. 2008; Kusuhara et al. 2018; American Diabetes Association 2021). Diabetic retinopathy (DR) is the most common microvascular complication of DM and one of the leading causes of preventable blindness. Worldwide, the number of people in 2020 with DR is estimated to have surpassed 100 million, or about 22% of the DM population (Teo et al. 2021). Among these, 28 million are considered to have vision-threatening diabetic retinopathy, defined as the presence of severe nonproliferative DR (NPDR), proliferative DR (PDR), clinically significant diabetic macular edema (DME), or a combination thereof.

Pathogenesis and Pathophysiology

Diabetes duration and sustained hyperglycemia are major risk factors associated with microvascular complications (Kusuhara et al. 2018). Sustained high levels of glucose can be deleterious to the neurovascular system, and although the exact mechanisms and pathways involved in the development of DR are not fully understood, several hypotheses have been proposed (Fig. 1) (Khalil 2017). Oxidative stress is believed to play a pivotal role and contribute to a cascade of other mechanisms (Giacco and Brownlee 2010). High levels of glucose lead to an increase in the generation of mitochondrial superanion oxidase and other reactive oxygen species, which can subsequently oxidate intracellular proteins. Overactivation of the polyol pathway leads to accumulation of sorbitol and osmotic imbalances. There is an increase in the production of advanced glycation end products (AGEs), as well as the receptors for AGEs (RAGEs), which are also deleterious for many cell structures. Additionally, hyperglycemia has been associated with hyperactivation of the protein kinase C (PKC) pathway and increases the expression of growth factors such as

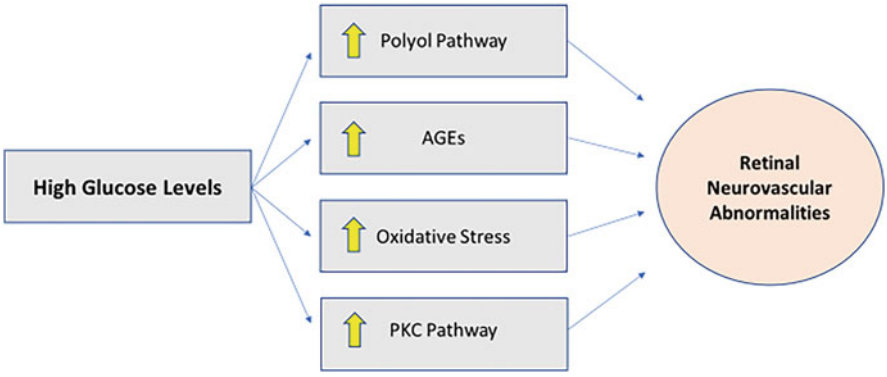

Fig. 1 Schematic presentation of potential mechanisms and pathways involved in the development of retinal neurovascular abnormalities in diabetic retinopathy Diabetic retinopathy (DR). Sustained high levels of glucose can lead to overactivation of the polyol pathway, hyperactivation of the protein kinase C (PKC) pathway, and an increase in the production of advanced glycation end products (AGEs) and reactive oxygen species

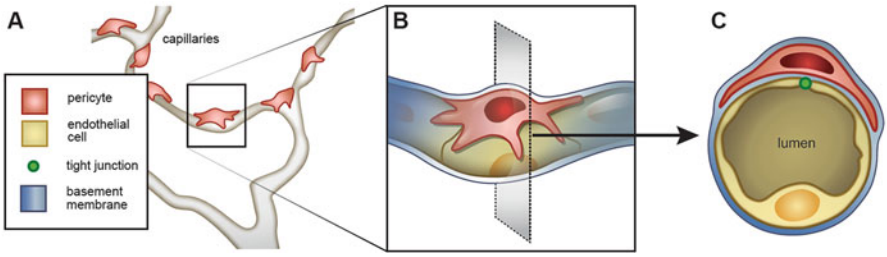

Fig. 2 Distribution of vascular endothelial cells and pericytes along the retinal capillaries. (**a**) Illustration of the retinal capillaries; (**b**) localization of pericytes on a retinal capillary; (**c**) capillary cross section showing the disposition of pericytes with tight junctions and the endothelial cells (EC), both wrapped by the basement membrane. Pericyte-EC crosstalk is essential for the homeostasis of retinal vasculature

vascular endothelial growth factor (VEGF) and many pro-inflammatory cytokines (Giacco and Brownlee 2010; Tarr et al. 2013; Kusuhara et al. 2018).

Stimulation of all these molecular pathways can contribute to the development of neurovascular abnormalities in the retina. Retinal vascular cells are comprised mainly of pericytes and endothelial cells. Retinal pericytes are specialized mural cells located on the abluminal side of the endothelial cells (ECs), and both cells are enveloped by the same basement membrane in retinal capillary vessels (Fig. 2) (Hammes et al. 2002; Trost et al. 2016; Liu et al. 2019).

Pericytes are important for vascular stability and for regulating microvascular blood flow and endothelial proliferation (Trost et al. 2016). Capillary ECs are connected by tight junctions and are essential for the inner blood-retina barrier, a barrier that restricts the flux of molecules and cells across the retinal capillaries to

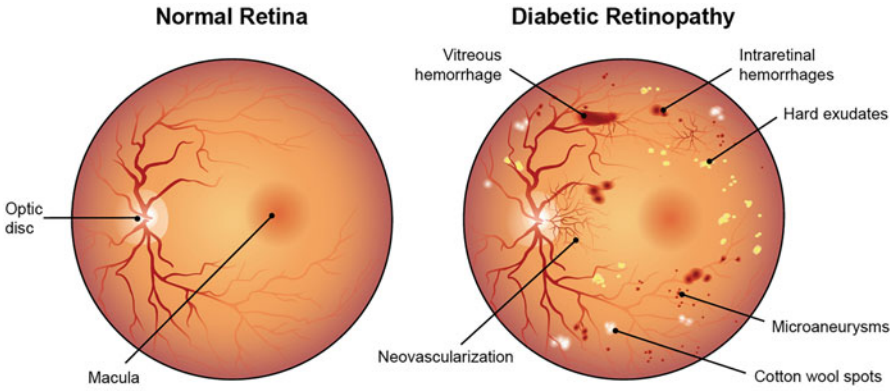

Fig. 3 Illustration of retinal fundoscopic findings in diabetic retinopathy. Left image shows some of the anatomical characteristics of the normal retina including the optic disc and macula. Right image shows pathologic signs commonly associated with diabetic retinopathy (DR), including microaneurysms, hard exudates, intraretinal hemorrhages, cotton wool spots, neovascularization, and vitreous hemorrhage. The last two signs are characteristic of severe stages of the DR

protect the neural environment (Campbell and Humphries 2012; Diaz-Coranguez et al. 2017; Fresta et al. 2020). Pericyte-EC crosstalk is crucial for the homeostasis of retinal vasculature (Liu et al. 2019).

Patients with DM present an increased loss of retinal pericytes, which over time can cause the formation of microaneurysms (localized outpouching of capillary walls), one of the hallmarks of early DR (Fig. 3). That process can be followed by the loss of endothelial cells, thickening of the basement membrane, and eventual occlusion of acellular capillaries, all of which are causes of increase in vascular permeability, impairment of the inner BRB, and leakage of lipids and proteins, forming hard exudates and macular edema. These vascular abnormalities can also lead to the formation of intraretinal hemorrhages and areas of ischemia and infarct of the nerve fiber layer (cotton wool spots) (Shin et al. 2014; Wang and Lo 2018). As the disease evolves, capillary occlusion and ischemia lead to upregulation of VEGF, which can promote even more increase in vascular permeability and the proliferation of new vessels (angiogenesis). Those neovessels are fragile and can easily rupture, leading to vitreous hemorrhage (Kroll et al. 2007).

Hyperglycemia can also be detrimental to the neural tissue of the retina, which leads to the concept that DR is a neurovascular disorder and that the dysfunction of the neural retina could even precede microvascular abnormalities (Nian et al. 2021).

Clinical Grading

The most common signs of DR can be observed with a simple ophthalmoscopic exam and are represented in Fig. 3. However, the gold standard classification of DR is based on a complex grading of stereophotographs of 7 fields and classifies DR into

Table 1 Severity Scale for Diabetic Retinopathy (based on Wilkinson et al. 2003)

NPDR
- Mild: Microaneurysms only
- Moderate: More than just microaneurysms but less than severe NPDR
- Severe: No signs of proliferative retinopathy but any of the following: More than 20 intraretinal hemorrhages in each of the four quadrants Definite venous beading in two or more quadrants Prominent IRMA in one or more quadrants
PDR
- One or both of the following: Neovascularization Vitreous/pre-retinal hemorrhage

NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; IRMA = intraretinal microvascular abnormality

13 levels ranging from level 10 (absence of retinopathy) to level 85 (severe vitreous hemorrhage or retinal detachment involving the macula) (ETDRS Research Group 1991; Wu et al. 2013). That grading system is known as the Diabetic Retinopathy Severity Scale (DRSS), and it was first used to assess DR severity in the Early Treatment Diabetic Retinopathy Study (ETDRS) (ETDRS Research Group 1991). It is a useful scale for clinical trials and is used in research settings by retina specialists, but it is too complex for daily clinical use. The International Clinical Disease Severity Scale for DR is a simplified classification commonly used for communication between ophthalmologists and other healthcare professionals, and it divides the disease into nonproliferative DR (mild, moderate, and severe) and proliferative DR based on retinal fundoscopic findings (Table 1) (Wilkinson et al. 2003).

More recently, other classification systems have been proposed for the research setting, using images obtained with more advanced technologies, such as ultra-widefield photography and optical coherence tomography (OCT), and some have demonstrated potential to identify subsets of patients at increased risk of progression of diabetic retinopathy (Aiello et al. 2019; Solomon and Goldberg 2019).

Chhablani et al. (2015) demonstrated a thinning of retinal ganglion cell inner plexiform layer by spectral domain OCT in DM type 2 subjects at all stages, including non-DR, which reinforces the concept that DM affects the complete neurovascular unit of the retina, and neurodegenerative changes may precede the visible vascular signs.

Clinical Management

Routine ophthalmological examinations are recommended for all diabetic patients, with intervals dependent on the severity of the disease (Solomon et al. 2017; Wong et al. 2018). In general, the American Academy of Ophthalmology recommends at least yearly screenings, starting at the time of diagnosis if DM type 2, or 5 years after diagnosis if DM type 1 (Flaxel et al. 2020). For patients with early disease (mild and

moderate NPDR), systemic treatment of hyperglycemia is critical in preventing or delaying the progression of DR (Kusuhara et al. 2018).

The UK Prospective Diabetes Study (UKPDS) showed that a 1% decrease in HbA1c (e.g., from 9% to 8%) was associated with a 35% reduction in the risk of microvascular complications, and the Diabetes Control and Complications Trial (DCCT) found that a 10% reduction in HbA1c (e.g., 8% vs. 7.2%) reduced the risk of DR progression by more than 40% (DCCT Research Group 1995; UKPDS Group 1998). More recently, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) eye study confirmed that although intensive glycemic control does not prevent retinopathy completely, it can significantly reduce the risk of the development and progression of diabetic retinopathy (Accord Study Group et al. 2010).

Blood pressure and serum lipid control are also recommended because some studies have suggested that reducing systolic pressure and using fenofibrate for dyslipidemia reduced the risk or slowed the progression of diabetic retinopathy (Sharma et al. 2015; Solomon et al. 2017).

In addition to systemic treatments, eyes with severe NPDR, PDR, or clinically significant DME can benefit from receiving local treatments. Laser photocoagulation used to be the gold standard treatment for all vision-threatening stages of DR. Panretinal photocoagulation (PRP) can reduce the risk of severe visual loss in eyes with PDR, for which it remains considered the mainstay treatment (Fig. 4), and focal/grid laser therapy is the preferred treatment for non-center-involved diabetic macular edema (Wang and Lo 2018; Flaxel et al. 2020). The mechanisms by which retinal laser photocoagulation works are not completely understood. The main hypothesis is that the destruction of hypoxic retina in regions outside of the central macula decreases the oxygen consumption and facilitates diffusion of oxygen from the choroid through the lesions created by PRP (laser scars). Both mechanisms can explain a reduction of hypoxia in the inner retina and consequently a downregulation in the production of angiogenic factors by ischemic

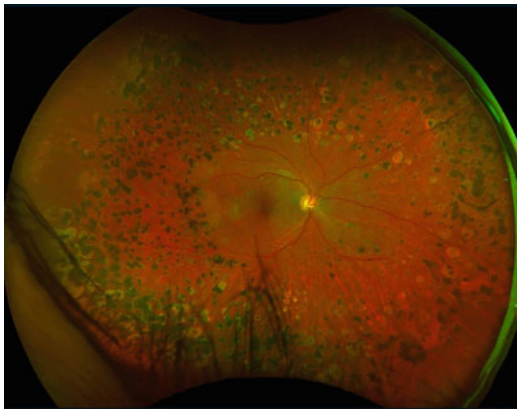

Fig. 4 Eye retinography with proliferative diabetic retinopathy (PDR) treated with laser panretinal photocoagulation (PRP). Widefield view of eye treated with PRP. Photocoagulation scars are located outside of the macula and approximately one-half burn width apart (UCSF Department of Ophthalmology)

tissue, which reduces the risk of neovascularization and attempts to preserve the central vision (Stefansson 2001).

Over the last two decades, intravitreal injections of anti-VEGF drugs have proven to be very effective in treating a variety of exudative retinal diseases, including DR. DME may occur at any stage of DR and is caused by the break in BRB and increased vascular leakage. Intravitreal anti-VEGF agents are very effective in the treatment of center-involved DME, and for these cases they are currently considered the first-line therapy, with possible deferred focal laser treatment (Diabetic Retinopathy Clinical Research et al. 2012; Flaxel et al. 2020). Given the relatively rapid improvement of DME after laser photocoagulation, it is believed that the main mechanism of action is the modulation of VEGF-induced vascular permeability (Kim and D'Amore 2012).

Patients with persisting or recurring DME, refractory to intravitreal anti-VEGF therapy, are presumed to have a more inflammatory profile with increased levels of multiple pro-inflammatory cytokines and are considered good candidates for treatment with intravitreal corticosteroids. Steroids are broad anti-inflammatory agents that act on multiple targets. The main reason for their not being preferred over anti-VEGF injections as the first line of therapy for DME is that steroid intravitreal injections and implants have higher risks of complications, including intraocular pressure elevation and cataract (He et al. 2018).

For treatment of PDR, the results of monotherapy with intravitreal anti-VEGF drugs have shown non-inferiority to PRP standard care (Writing Committee for the Diabetic Retinopathy Clinical Research et al. 2015). In the CLARITY trial, treatment of PDR with intravitreal injections of aflibercept, which is a vascular endothelial growth factor-A (VEGF-A) and placental growth factor (PGF) antagonist, was associated with improved outcomes at 1 year versus patients treated with PRP (Sivaprasad et al. 2017). In eyes with PDR and DME, many specialists recommend the combination of either intravitreal anti-VEGF or steroid and PRP (Giovannini et al. 2019). Lastly, in advanced cases with vitreous hemorrhage, surgical vitrectomy may be required.

As explained above, the decision to start systemic treatments is based mainly on blood biomarkers (e.g., HbA1c), and the choice of local eye treatments is based mainly on changes observed in the retinal structure and imaging biomarkers. Unfortunately, after three to six monthly injections, more than 1/3 of the patients receiving anti-VEGF injections to treat DME are classified as poor responders or non-responders (Bressler et al. 2018; Maggio et al. 2018). Currently there is no good way to predict who could benefit more from using an alternative treatment as first line.

There is a great need to discover new therapeutic targets to treat patients that do not respond well to any of the current available therapies and to find new biomarkers that are prognostic of DR progression and predictive of the best choice of clinical treatment for an individual. With the recent advancements in proteomics and the development of multiplexed platforms capable of working with very small volumes of specimen, the search for local new molecular biomarkers in ocular specimens such as the vitreous and aqueous humor became very promising, and many proteins are currently being investigated.

Assessing Local Molecular Biomarkers for DR

The term biopsy originates from the Greek words *bios* (life) and *ópsis* (sight). It is the medical procedure of extracting cells or tissues to be examined for disease.

Retinal biopsies are not considered a reasonable option for researching DR biomarkers in live patients because they carry great comorbidity, and therefore their use is reserved in ophthalmology for selected cases of atypical retinitis, uveitis, or neoplasms (Westerfeld and Mukai 2009). Biopsies of the ocular fluids are safer options, and the location adjacent to the retina makes the vitreous a great source of molecular biomarkers for posterior segment disorders, including DR. In fact, high levels of many proangiogenic and pro-inflammatory cytokines have been found in the vitreous of patients with DR (Banerjee et al. 2007; Agrawal et al. 2014; Dai et al. 2014; Lamy et al. 2020).

Ocular Liquid Biopsy

Vitreous is a gel matrix consisting mainly of water, collagen, and hyaluronan, and it fills the posterior chamber of the eye, being surrounded by the retina, ciliary body, and lens (Bishop 2000; Sebag 2012; de Smet et al. 2013). Vitreous biopsies can be obtained by needle aspiration inserting a 23- to 25-gauge needle around 4 mm behind the limbus through the conjunctiva, sclera, and pars plana into the vitreous chamber (Fig. 5). This technique has some disadvantages as the volume aspirated is usually very limited and since the vitreous is not homogeneous, some authors hypothesize that only the less viscous and more liquid portion, containing soluble proteins, would be collected. There is another option which is preferred by most surgeons, and it consists of collecting vitreous using a vitreous cutter, a small, high-speed guillotine instrument. This is usually done as part of a surgical procedure and as such is required to be done under proper anesthesia in the operating room. The advantage is that the surgeon can chop and aspirate even the more solid parts of the vitreous and obtain a larger sample volume with lower incidence of hypotony, since the surgical infusion is initiated immediately after the vitreous collection, stabilizing the intraocular pressure through the rest of the surgical procedure (Skeie et al. 2012; Velez et al. 2018).

Skeie et al. (2012) compared vitreous samples collected with both methods and found that the concentration of total protein was very similar whether biopsies were obtained with a needle or vitreous cutter instrument, although a minority of proteins and peptides differed in concentration.

An even safer option is to aspirate aqueous humor (AH) from the anterior chamber. The procedure can be done in an office-based setting under local anesthesia. Proteins secreted by the retina into the vitreous can diffuse into the aqueous humor, and the levels of many cytokines in the AH were found to correlate with the vitreous levels in patients with DR (Funatsu et al. 2005; Wu et al. 2020).

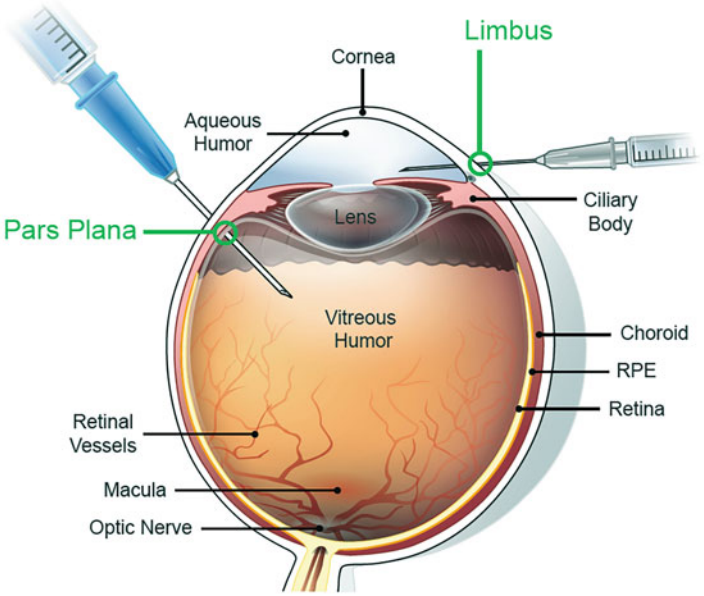

Fig. 5 Illustration of eye anatomy including the approximate insertion locations used to obtain liquid biopsies. A thin needle can be inserted into the anterior chamber, near the corneal limbus, for aspiration of aqueous humor. To access the vitreous humor, a needle or a vitreous cutter can be inserted 3.5–4 mm behind the limbus, through the pars plana, with the tip pointing to the center of the eye to reduce the risks of lens and retinal damage

Sample Selection and Method of Analysis

When comparing results from different studies, it is important to consider that some aspects of the vitreous collection and patient selection (inclusion and exclusion criteria) can have an influence on the vitreous composition. For instance, some studies use a hemoglobin concentration cutoff to exclude vitreous samples containing blood from vitreous hemorrhage because those samples are likely to have an enormous concentration of proteins and confound the results. Likewise, history of recent intravitreal injections or laser photocoagulation treatment on the retina can significantly affect cytokine levels in the vitreous and needs to be noted (Shimura et al. 2007; Adamiec-Mroczek and Oficjalska-Mlynczak 2008; Shimura et al. 2009; Suzuki et al. 2020). Also, it has been shown that the level of analytes may vary according to the location of collection, and the levels of cytokines like VEGF are higher in the pre-macular vitreous than in the center of the vitreous body (Shimada et al. 2009).

The selection criteria used for the control group is also important, as it is usually comprised of samples obtained from patients undergoing ocular surgical procedures for other diseases, such as epiretinal membrane and macular hole.

The analysis of vitreous samples from DR patients can be performed using untargeted methods based on mass spectrometry for study of metabolomics or proteomics (Loukovaara et al. 2015; Patnaik et al. 2019; Tamhane et al. 2019) or targeted methods, such as enzyme-linked immunosorbent assays (ELISAs). Untargeted methods can identify thousands of analytes and generate large datasets that may require a more complex analysis and use of bioinformatics. On the other extreme, traditional singleplex ELISAs may be performed using straightforward commercially available kits and can generate accurate data, but they only measure one analyte per sample which makes them impractical for biomarker screening (Aydin 2015; Tighe et al. 2015). Multiplexing technologies have significantly advanced over the last decades, and many platforms have been successfully applied to study vitreous from DR patients, allowing for the detection of hundreds of proteins of interest using minute volumes of vitreous (Lamy et al. 2020). Figure 6 illustrates the mechanisms used by two popular multiplex platforms: electrochemiluminescent (ECL) sandwich immunoassay and proximity extension assay (PEA).

Most commercially available multiplex platforms were developed aiming to test blood plasma samples. Differences in protein abundance between plasma and vitreous and the nonspecific matrix background can affect the detection rate of individual analytes. When designing a vitreous study, the best method to choose may be dependent on the main targets of interest, since one platform may work better than the others for specific vitreous analytes. The authors previously reported the detection rates of many vitreous analytes using PEA and ECL platforms (Table 2).

Vitreous Molecular Biomarkers of DR

Changes in the vitreous molecular composition have provided valuable insight into the pathogenesis, diagnosis, and treatment of retinal diseases (Tamhane et al. 2019). Numerous vitreous molecular biomarkers have been associated with DR, and some of the most studied markers are discussed below.

Cytokines

Cytokines (derived from the Greek words *kyto,* which means cell,) and *kineo,* which means "to move") are a broad category of messenger molecules that aid cell-to-cell communication. Some cytokines can regulate leukocyte migration and are called chemokines; others are called growth factors because they can trigger cell proliferation and/or differentiation. The term cytokine also encompasses interferons, interleukins, adipokines, and the tumor necrosis factor family (Dinarello 2007). Vitreous cytokines have long been associated with DR (Abu el Asrar et al. 1992; Adamis et al. 1994), and hundreds of studies have been published over the last decades.

In a meta-analysis of 389 datasets from 118 articles, McAuley et al. (2014) found that 154 analytes (mostly cytokines) were identified in association with DR, and 12 of them had been reported in more than 3 independent studies comparing

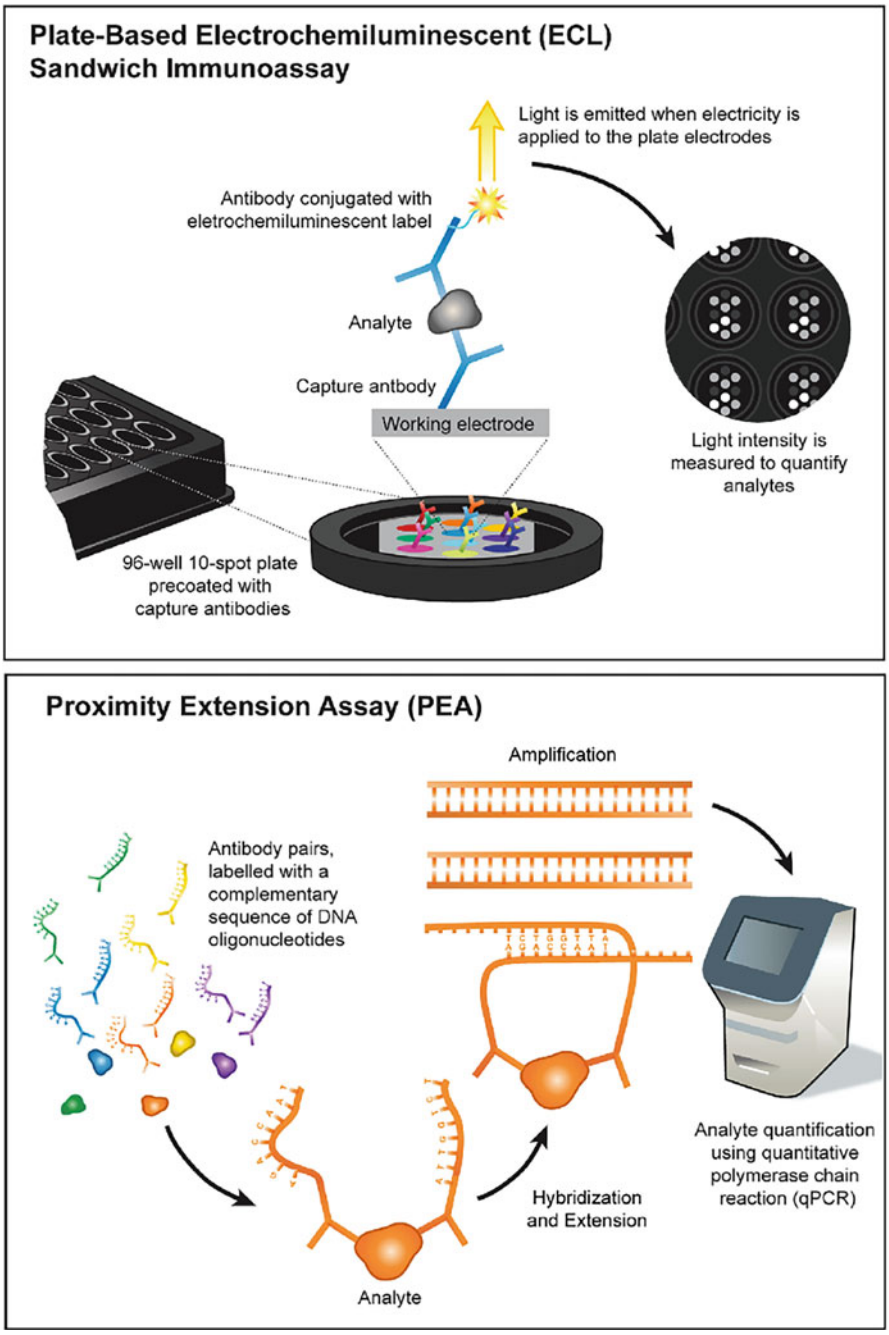

Fig. 6 Illustration of the mechanisms used by two multiplexed platforms: proximity extension assay (PEA) and electrochemiluminescent (ECL) assay. (Reprinted with permission from Lamy et al. 2020 Copyright 2020 The Authors – licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License)

Table 2 Comparison of detection rates of numerous vitreous analytes using PEA and ECL platforms. (Reprinted with permission from Lamy et al. 2020 Copyright 2020 The Authors – licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License)

	Platform					
	ECL			PEA		
		Detection (%)			Detection (%)	
Protein	Panel	Control	PDR	Panel	Control	PDR
Eotaxin	CH	0.0	0.0	IN	100.0	100.0
FGF2	AN	20.0	7.1	IO	23.5	5.0
ICAM-1	VI	100.0	100.0	CM	70.6	100.0
IFN-γ	PI	0.0	0.0	IO	11.8	5.0
				IN	0.0	0.0
IL-1α	CY	11.8	25.0	IO	0.0	0.0
				IN	0.0	0.0
IL-2	PI	0.0	5.0	IO	0.0	10.0
				IN	0.0	0.0
IL-4	PI	0.0	5.0	IO	17.6	15.0
				IN	5.9	5.0
IL-5	CY	0.0	0.0	IO	0.0	0.0
				IN	0.0	0.0
IL-6	PI	94.1	100.0	IO	100.0	100.0
				IN	100.0	100.0
IL-7	CY	100.0	100.0	IO	100.0	100.0
				IN	100.0	100.0
IL-8	CH	0.0	5.3	IO	100.0	100.0
	PI	100.0	100.0	IN	100.0	100.0
IL-10	PI	0.0	5.0	IO	11.8	50.0
				IN	0.0	25.0
IL-12p40	CY	94.1	100.0	IN	100.0	100.0
IL-12p70	PI	0.0	0.0	IO	100.0	100.0
IL-13	PI	0.0	0.0	IO	5.9	5.0
				IN	5.9	5.0
IL-17A	CY	0.0	0.0	IN	17.6	25.0
IP-10	CH	100.0	100.0	IO	100.0	100.0
				IN	100.0	100.0
MCP-1	CH	100.0	100.0	IO	100.0	100.0
				IN	100.0	100.0
MCP-4	CH	0.0	10.5	IO	100.0	100.0
				IN	5.9	45.0
MIP-1α	CH	0.0	10.5	IO	100.0	100.0
				IN	100.0	100.0
MIP-1β	CH	100.0	100.0	IO	100.0	100.0
				IN	100.0	100.0
PGF	AN	0.0	100.0	IO	100.0	100.0

(continued)

Table 2 (continued)

	Platform					
	ECL			PEA		
		Detection (%)			Detection (%)	
Protein	Panel	Control	PDR	Panel	Control	PDR
TARC	CH	0.0	42.1	IO	100.0	100.0
Tie-2	AN	0.0	0.0	IO	100.0	100.0
TNF-α	PI	0.0	30.0	IO	0.0	0.0
				IN	5.9	5.0
TNF-β	CY	0.0	5.0	IN	76.5	95.0
VCAM-1	VI	70.6	94.7	CM	23.5	50.0
VEGF-A	AN	26.7	100.0	IO	100.0	100.0
	CY	6.3	90.0	IN	100.0	100.0
VEGF-C	AN	6.7	7.1	IO	94.1	95.0
VEGF-D	AN	0.0	35.7	CR	88.2	100.0

ECL, electrochemiluminescent sandwich immunoassay; PEA, proximity extension assay; PDR, proliferative diabetic retinopathy; panels: PI, pro-inflammatory; CY, cytokine; CH, chemokine; VI, vascular, AN, angiogenesis; IO, immuno-oncology; IN, inflammation; CM, cardiometabolic; CR, cell regulation

nondiabetics with PDR. Out of the 12, pigment epithelium-derived factor (PEDF) and hepatocyte growth factor (HGF) were significantly decreased in PDR samples, and the other 10 analytes were found to be significantly increased in PDR samples: VEGF, interleukin-6 (IL-6), interleukin-8 (IL-8), erythropoietin (EPO), platelet-derived growth factor BB chain (PDGF-BB), nitric oxide (NO), endothelin-1 (ET-1), monocyte chemotactic protein 1 (MCP-1), transforming growth factor beta (TGF-β), and tumor necrosis factor alpha (TNF-α).

VEGF, also called VEGF-A, is the parental member of a family of related growth factors that share structural features but have different biological specificities and includes PGF, VEGF-B, VEGF-C, VEGF-D (initially known as c-Fos-induced growth factor, FIGF), and VEGF-E (Li and Eriksson 2001; Holmes and Zachary 2005). VEGF is probably the most studied angiogenic factor in vitreous of eyes with DR, with more than 30 studies reporting increased levels of VEGF in PDR vitreous compared to controls. VEGF was also found to be significantly increased in at least three studies comparing NPDR vitreous to nondiabetic controls. PGF may promote angiogenesis indirectly or directly through a VEGF receptor, and high levels of PGF have been reported in vitreous of PDR eyes (Mitamura et al. 2002; Tsai et al. 2018).

Using an ECL multiplex assay to study vitreous samples from PDR and control eyes, Lamy et al. (2020) observed that among the analytes found to be significantly increased in PDR samples, VEGF presented the highest magnitude of difference, followed by IL-8 and IL-6.

IL-8 (or CXCL8) is a pro-inflammatory chemokine that is involved in the recruitment of granulocytes (mainly neutrophils) and is also known for promoting inflammation-mediated angiogenesis. IL-8 can be secreted by cells that are involved in innate immune response, such as macrophages, and it can also be overexpressed

by retinal endothelial and glial cells in response to hypoxia. High levels of IL-8 in the vitreous have been associated with DME and with subretinal fluid formation in DR (Yenihayat et al. 2019), suggesting that inflammation can be an important component in the progression of DME. Petrovic et al. (2010) have observed that among patients with PDR undergoing vitrectomy surgery, elevated vitreous levels of IL-8 at the time of surgery were associated with poor visual outcome after the procedure. They suggested that IL-8 could be a biomarker of ischemic inflammatory reaction and possibly contribute to deterioration of visual acuity and DR progression.

Using a flow cytometry-based multiplex assay to compare the levels of 17 analytes in the vitreous and plasma of eyes with PDR and nondiabetic controls, Koskela et al. (2013) found that in patients with PDR, the concentrations of IL-8 and IL-6 were, respectively, 589% and 2613% higher in the vitreous than in the plasma, suggesting that those interleukins are being produced mainly by the retina and local ocular tissues rather than originating from systemic inflammation. Also, when compared to controls, the vitreous concentration of IL-8 and IL-6 in PDR eyes was more than 65 times higher, corroborating the finding that those cytokines are associated with PDR and that immune-inflammatory pathways can play a role in the DR disease progression. The use of anti-IL8 medications has been explored pre-clinically for ocular indications, and the investigation of a monoclonal antibody anti-IL8 has reached clinical stage for indications in the oncology field (Bilusic et al. 2019).

IL-6 (formerly known as B cell-stimulating factor) is a pleiotropic chemokine with both pro- and anti-inflammatory characteristics with key roles in multiple biological processes, including the immune and inflammatory response. In a pooled analysis including 48 studies in DR, Ulhaq et al. (2020) found that IL-6 levels in intraocular fluids (vitreous and aqueous) were significantly higher in DR than in controls, and when subgroups were compared, the NPDR group showed lower IL-6 levels than the PDR group, suggesting an association with disease severity.

MCP-1, also known as chemokine ligand 2 (CCL2), is a chemotactic factor for monocytes and macrophages and has been implicated in the pathogenesis of many diseases including DR. Hyperglycemia promotes MCP-1 expression under the control of nuclear factor kappa B (NF-kB) (Harada et al. 2006). In patients with DR, there is an increased expression of MCP-1 in the eye by retinal pigment epithelial cells (RPE), vascular endothelial cells, and glial cells (Taghavi et al. 2019). Urbancic et al. (2020) found a 22 times higher concentration of MCP-1 in the vitreous when compared to serum of patients with PDR, confirming the importance of the local intraocular secretion of MCP-1.

Erythropoietin (EPO) stimulates the production of mature red blood cells (RBC) and is an essential growth factor for the erythrocyte lineage (Jelkmann 1992). EPO production can be induced by hypoxia, and it has angiogenic properties, with activity in vascular endothelial cells. Watanabe et al. (2005) found 12 times higher levels of EPO in vitreous of PDR eyes than in nondiabetic controls, suggesting it may have a role in PDR. Also, the absence of correlation found between the vitreous and plasma levels of EPO suggested that the higher levels in the vitreous were due to local production in the eye. Similarly, Katsura et al. (2005) reported EPO vitreous

concentration levels (normalized to total protein concentration) to be more than eight times higher in PDR than in samples collected from the eyes with macular hole.

Hepatocyte growth factor (HGF) is a paracrine growth factor secreted by mesenchymal cells which acts via tyrosine-protein kinase Met (c-Met) receptor and regulates cell motility and growth. HGF can act dually as a pro- and anti-inflammatory mediator and as a pro-angiogenic factor. Mixed results have been reported in regard to DR. Katsura et al. (1998) found levels of HGF two times higher in the vitreous of patients with PDR compared to nondiabetic controls. Nishimura et al. (1999) found HGF levels almost four times higher in vitreous from eyes with PDR compared to nondiabetic controls, and among eyes with PDR, the levels were 63% higher when iris neovascularization was present, a sign associated with extensive areas of ischemia in the retina. McAuley et al. (2014) reported lower levels of HGF in PDR vitreous compared to nondiabetic controls. The same group also reported lower PEDF levels in vitreous samples from eyes with DME and/or PDR. PEDF is a multifunctional protein that can be secreted by numerous types of cells including vascular EC, RPE, glial cells, and neurons. PEDF presents anti-angiogenic and neuroprotective functions and may inhibit oxidative stress and inflammation (Nian et al. 2021).

Angiopoietins 1 to 4 (Ang 1–4) are members of a family of growth factors, whose activity is mediated by the tyrosine kinase receptors, Tie1 and Tie2 (Akwii et al. 2019). Significantly higher levels of Ang2 have been reported in vitreous of eyes with DR (Watanabe et al. 2005; Tuuminen et al. 2015; Lamy et al. 2020). Ang2 is thought to have a role in vascular permeability, and a novel promising intravitreal drug (faricimab) targeting Ang2 and VEGF-A for treatment of DME is currently under investigation (Heier et al. 2021).

The interactions of 12 significant biomarkers in PDR are presented in Fig. 7 (McAuley et al. 2014). The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org) pathway analysis showed the central importance of VEGF, as it can be activated by several proteins including IL-6, IL-8, HGF, EPO, and TNF-α.

Kinin-Kallikrein System (KKS)

KKS is an endogenous multiprotein cascade system with multiple roles in inflammation, vasodilation, vascular permeability, and coagulation (Kashuba et al. 2013). The effects of this system are mainly mediated by bradykinin (BK), a peptide hormone with potent pro-inflammatory and vasodilatory effects. Two serine protease zymogens (inactive proenzymes) are involved in the generation of BK after activation: plasma prekallikrein (PPK) and coagulation Factor XII (FXII). FXII can be activated upon interaction with an activating surface into FXIIa. The substrates of FXIIa include FXI, involved in activation of intrinsic coagulation, and PPK, resulting in the production of plasma kallikrein (PK). The proteolytically active PK can activate additional FXII and also cleave high-molecular-weight kininogen (HK), resulting in the release of BK (Fig. 8) (Phipps and Feener 2008).

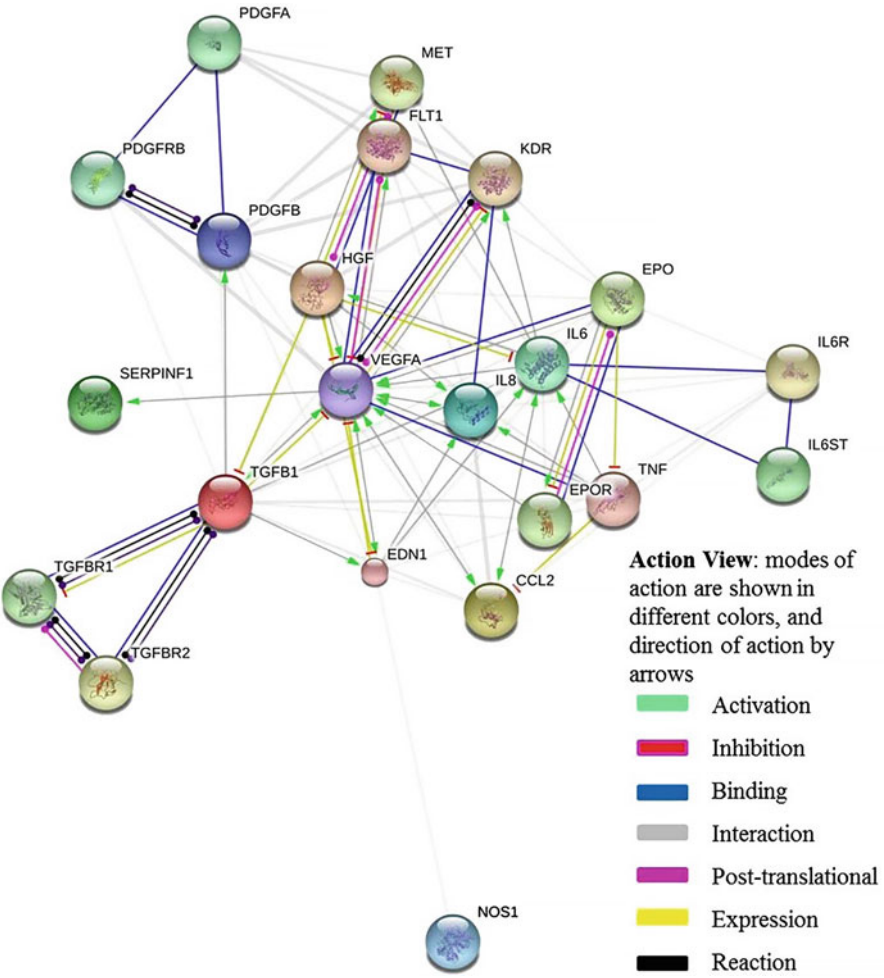

Fig. 7 Molecular pathway analysis of significant analytes in proliferative diabetic retinopathy (PDR). STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) pathway analysis. Abbreviations: CCL2 (MCP-1), monocyte chemotactic protein 1; EDN1 (ET-1), endothelin-1; EPO, erythropoietin; EPOR, erythropoietin receptor; FLT1, vascular endothelial growth factor receptor 1; HGF, hepatocyte growth factor; IL6, interleukin-6; IL8, interleukin-8; IL6R, interleukin-6 receptor; IL6ST, interleukin-6 receptor subunit beta; KDR, vascular endothelial growth factor receptor 2; MET, hepatocyte growth factor receptor; NOS1 (NO), nitric oxide; PDGFA, platelet-derived growth factor subunit A; PDGRB, platelet-derived growth factor BB chain; PDGFRB, platelet-derived growth factor receptor beta; SERPINF1 (PEDF), pigment epithelium-derived factor; TGFB1 (TGF-β), transforming growth factor beta 1; TGFBR1, transforming growth factor beta receptor 1; TGFBR2, transforming growth factor beta receptor 2; TNF-α, tumor necrosis factor-alpha; VEGFA, vascular endothelial growth factor A (Reprinted from Journal of Diabetes and its Complications, Vol 28.3, McAuley, Annie K., et al. Vitreous biomarkers in diabetic retinopathy: a systematic review and meta-analysis. Pages 419–425, Copyright 2014, with permission from Elsevier)

Kita et al. (2015) found a twofold increase in PPK and an 11-fold increase in PK in vitreous samples from eyes with DME compared with those with a macular hole (MH). Loukovaara et al. (2015) found that five protein components of the KKS were increased in vitreous of patients with PDR compared with NPDR: FXII, kallikrein B1 (KLKB1), alpha 2-antiplasmin (A2AP), kininogen 1 (KNG1), and plasminogen (PLMN). They suggested that the enrichment of those proteins observed in the disease progression from NPDR to PDR was confirming the importance of KKS in the pathogenesis of DR. Numerous small molecules and peptides targeting KKS are currently under investigation for DME treatment; some are for oral and topical administration, which could represent an advantage over intravitreal injections (Bhatwadekar et al. 2020).

Complement System (CS)

The CS functions as the first defense line of innate immunity, and it is composed of three proteolytic pathways: classical, lectin, and alternative pathways. CS has been implicated in the pathogenesis of several diseases including DR. Loukovaara et al.

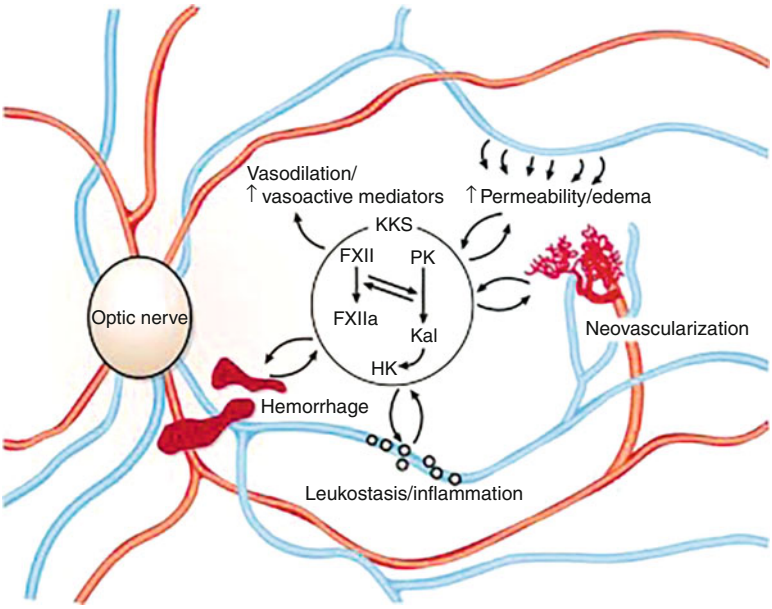

Fig. 8 Schematic illustrating the potential involvement of the KKS in diabetic retinopathy. FXII, factor XII; FXIIa, factor XIIa; HK, high-molecular-weight kininogen; Kal, kallikrein; PK, prekallikrein; KKS, kallikrein-kinin system. (Reprinted from Kidney international 73, no. 10, Phipps, J. A., and E. P. Feener. The kallikrein–kinin system in diabetic retinopathy: lessons for the kidney. Pages 1114–1119, Copyright 2008, with permission from International Society of Nephrology)

(2015) detected 20 complement cascade components in vitreous samples of both PDR and NPDR eyes and found that 15 of them were significantly higher in the vitreous of PDR eyes compared to NPDR, suggesting the presence of CS proteins since the early stages of DR and an increase in more severe cases.

Using albumin and complement levels from plasma to normalize the vitreous data and control for vascular leakage of proteins from the systemic circulation to the vitreous cavity, Mandava et al. (2020) reported an increased activation through the alternative complement pathway in PDR eyes, with local activation of complement factors C3a, C5a, and Ba, as well as local consumption of C4, C5, and factor B.

Shahulhameed et al. (2020) also found signs of increased CS activation through the alternative pathway with high levels of C3 and its activated fragment C3ba' in the vitreous of PDR eyes.

MicroRNA (miRNA)

MiRNAs are sequences of small noncoding nucleotides that play important roles in gene expression. The expression of vitreal miRNAs is found to be altered in many vitreoretinal diseases including DR (Ragusa et al. 2013; Hirota et al. 2015; Gomaa et al. 2017). Friedrich et al. (2020) found the expression of six miRNAs (hsa-miR-20a-5p, hsa-miR-23b-3p, hsa-miR-142-3p, hsa-miR-185-5p, hsa-miR-326, and hsa-miR-362-5p) to be elevated in the vitreous of PDR patients compared with nondiabetic controls undergoing vitrectomy for macular hole. Two of the miRNAs (hsa-miR-20a-5p and hsa-miR-185-5p) have VEGF-A as a validated gene target, and the other four are predicted to target genes of proteins associated with angiogenesis and wound healing responses. Anti-miRNAs may have therapeutic potential for DR.

Metabolites

Metabolites are small molecules (<1.5 kD) and represent the intermediate or end products of metabolism. They can be endogenous (gene-derived metabolites) or exogenous metabolites (environmentally derived metabolites). Haines et al. (2018) studied the vitreous of PDR eyes using untargeted mass spectrometry-based metabolomics and found a significant decrease in xanthine, a purine metabolite, and an increase of related purines (inosine, hypoxanthine, urate, allantoate). Together, those findings may suggest an upregulation in the enzyme xanthine oxidase, which is a superoxide-producing enzyme.

Applications to Prognosis and Other Diseases and Conditions

In this chapter, the use of vitreous molecular biomarkers to study DR was reviewed, with emphasis on the potential to determine new targets for drug discovery, as well as on the possibility to be used in precision medicine as predictive biomarkers,

impacting the clinical management of individuals or subgroups of patients with DR. More than 70 years ago, Michaelson proposed that a "factor X," produced by the retina, could promote neovascularization (Michaelson 1948). Five decades later, VEGF was discovered and found to be increased in the vitreous of diabetic patients (Leung et al. 1989; Adamis et al. 1994; Aiello et al. 1994). Intravitreal anti-VEGF drugs have revolutionized the treatment of DR and other retinal diseases, such as age-related macular degeneration (AMD) and retinal vein occlusions. With the advancement of the scientific methods, it is now known that there are more "factors" in the vitreous than letters in the alphabet. Over the years, researchers in academia and industry have been dedicating a lot of effort to identify the best prognostic and predictive vitreous biomarkers for DR, and numerous promising drugs are currently under pre-clinical and clinical investigation stages.

Key Facts of Vitreous Biomarkers in Diabetic Retinopathy (DR)

- The global number of people with DR in 2020 was estimated at 103.12 million, and it is projected to rise to 160.50 million in 2045.
- According to the US Centers for Disease Control and Prevention (CDC), DR is the leading cause of blindness among the American adult population (20–74 years old).
- To date, thousands of analytes have been identified in the human vitreous, and hundreds of them are increased in the eyes with DR, especially in the proliferative stage.
- Vascular endothelial growth factor is a key player in the pathogenesis of DR.
- Pegaptanib was the first anti-VEGF drug approved by the FDA for intra-ocular use in 2004 (indication: neovascular AMD), and ranibizumab was the first anti-VEGF to receive FDA approval for DR in 2012 (indication: diabetic macular edema).
- More than 1/3 of patients with DR do not respond to anti-VEGF therapy.
- Knowledge about vitreous biomarkers has supported the development of numerous drug candidates targeting a variety of molecular pathways for treatment of retinal diseases.

Mini-Dictionary of Terms

- **Blood-retina barrier (BRB)** – composed of an outer BRB, in which RPE cells regulate the transport between the choroid capillaries and the retina, and an inner BRB, which regulates movement of molecules and cells between retinal capillaries and retina.
- **Drug Discovery** – the process of discovering and developing new medicines to treat diseases or medical conditions. Modern drug discovery usually involves the screening of large compound libraries against isolated biological targets. The

study of biomarkers in a specific disease can facilitate the identification of potential new targets.

- **Limbus** (also known as corneoscleral junction) – border zone between the transparent cornea and the opaque sclera.
- **Macula** – central area of the retina responsible for detailed vision.
- **Optic disc** (also known as optic nerve head) – oval area in the nasal retina where the retinal ganglion cells exit the eye to form the optic nerve. It is also the entry point for the major blood vessels of the retina.
- **Pars plana** – relatively avascular posterior flat portion of the ciliary body that extends from the ciliary processes to the periphery of the retina. The location is commonly used for incisions to access the vitreous chamber during intra-ocular procedures.
- **Precision medicine** (also referred to as personalized medicine) – model where the medical provider tailors medical decisions and disease treatments based on patient genetics or biomarker information.
- **Predictive biomarker** – used to identify individuals who are more likely than others to achieve a favorable or unfavorable effect from a specific medical treatment or exposure to an environmental agent.
- **Prognostic biomarker** – used to identify likelihood of disease progression or related clinical events in individuals with the disease of interest.

Summary Points

- Vitreous humor contains thousands of proteins released by the retina and nearby structures, making it a great source for biomarkers of retinal diseases.
- Diabetic retinopathy (DR) is a common complication of diabetes mellitus and affects more than 100 million people worldwide.
- Intravitreal administration of anti-VEGF drugs and corticosteroids has revolutionized the treatment of vision-threatening stages of DR.
- More than 1/3 of patients treated with anti-VEGF therapy are considered non-responders.
- Novel prognostic and predictive biomarkers in DR have potential to facilitate disease prognosis, predict the best responders to a specific treatment, and support the development of new therapies.

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Protein Pyrrole Adducts in Diabetes Mellitus

48

Links with Axonal Polyneuropathy

Xiao Chen, Zhuyi Jiang, and Peter S. Spencer

Contents

Introduction 993
Diabetic Distal Symmetrical Polyneuropathy 994
DSPN: Central-Peripheral Distal Axonopathy 995
DSPN Etiology and Pathogenesis 996
Existing DSPN Biomarkers 998
Protein Pyrrole Adducts 999
Cross-Linking of γ-Diketone-Derived Pyrroles 1003
Cross-Linking of Protein Pyrrole Adduct: Links with DSPN 1006
Detection of Pyrrole Adducts 1007
Applications to Peripheral Neuropathy 1008
  Occupational *n*-Hexane/2-Hexanone Neuropathy 1008
  Diabetic Polyneuropathy 1009
Conclusions 1009
Mini-Dictionary of Terms 1010
Key Facts 1010
Summary Points 1011
References 1012

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Abstract

Diabetes mellitus is commonly associated with a symmetrical, distal sensory or sensorimotor axonal polyneuropathy that develops insidiously and advances in accord with the hyperglycemic state. Elevated blood glucose promotes anaerobic glycolysis which in turn produces excess advanced glycation end products, many of which have a pyrrole structure that can react with critical neuroproteins. Pyrroles also form between endogenous γ-diketones, notably 2,5-hexanedione, and the ε-amine group of lysine residues of proteins, including those required for axonal maintenance. The pyrrolated proteins undergo oxidation and cross-linkage, which interferes with axonal transport of materials from sites of neuronal synthesis to distal destinations. Thus, the distal ends of elongate sensory and motor axons are first affected, which triggers distal nerve fiber degeneration. Comparable pathological changes in long tracts of the spinal cord result in an overall picture of central-peripheral distal axonopathy. Whether, which, and what levels of pyrrolated serum/urinary proteins are useful biological markers of diabetic polyneuropathy is the principal subject of this chapter.

Keywords

Advanced glycation end products · Cross-linked pyrrolated proteins · Central-peripheral distal axonopathy · 2,5-Hexanedione

Abbreviations

AA	Acetoacetate
ACHD	3-Acetyl-2,5-hexanedione
AFGP	Alkyl formylglycosyl pyrrole
AGE	Advanced glycation end product
CNS	Central nervous system
COVID-19	SARS-CoV-2-induced disease
CPDA	Central-peripheral distal axonopathy
DEHD	3,4-Diethyl-2,5-hexanedione
DiPHD	3,4-Diisopropyl-2,5-hexanedione
DM	Diabetes mellitus
DM-1	DM type 1
DM-2	DM type 2
DMAB	*p*-Dimethylaminobenzaldehyde
DMHD	3,4-Dimethyl-2,5-hexanedione
DPP-4	Dipeptidyl peptidase 4
DSPN	Distal sensory/sensorimotor peripheral neuropathy
GDM	Gestational DM
HbA1c	Hemoglobin A1c
3-HHD	3-Hydroxy-2,5-hexanedione
4-HNE	4-Hydroxy-2-nonenal
HO-1	*Heme oxygenase 1*

ICAM-1	Intercellular adhesion molecule 1
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-1RA	Interleukin 1 receptor antagonist
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MCP-1	*Monocyte chemoattractant protein 1*
MG	Methylglyoxal
miRNA	Microribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	Nuclear factor, erythroid 2-like 2
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
PARP-α	Poly[ADP-ribose]polymerase 1
PNS	Peripheral nervous system
RAGE	Receptor for AGE
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sICAM-1	Soluble intercellular adhesion molecule 1
SIRT1	Sirtuin 1
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRPV1	Transient receptor potential cation channel subfamily V member 1
VEGF	Vascular endothelial growth factor

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease of people of all ages and may appear de novo as gestational diabetes (GDM) during pregnancy. In 2019, DM was estimated to affect almost one half billion people worldwide, with projected increases of ~25% by 2030 and ~50% by 2045. DM prevalence in high-income nations exceeds that in low-income countries (10.4% vs. 4.0%) and is much higher in urban than rural regions (10.8 vs. 7.2%) (Saeedi et al. 2019). DM prevalence increases with age, as illustrated by disease prevalence doubling among South Korean adults aged >65 years (28%) vs. those aged ≥ 30 years (13.8%). DM comorbidities include obesity (53.2%), hypertension (61%), hypercholesterolemia (72%), and both hypertension and hypercholesterolemia (43.7%) (Jung et al. 2021). In the Chinese province of Zhejiang, older women and urban residence were associated with a higher annual prevalence of GDM, but growth between 2016 (6.02%) and 2018 (7.94%) was much greater in rural than urban areas (11.28% vs. 0.00%) (Wang et al. 2021). The global estimated age-standardized incidence rate for both DM type 1 (DM-1) and DM type 2 (DM-2) grew between 1990 and 2017, with the largest increase in high-income countries (Liu et al. 2020).

DM-1, which accounts for 5–10% of DM cases, is an autoimmune, insulin-dependent disorder of juvenile onset, although a latent-onset form may affect 2–12% of adults with DM (Banday et al. 2020). DM-2, a non-autoimmune form of DM that constitutes ~90–95% of cases, whether or not overweight or obese, is characterized by insulin resistance and pancreatic beta-cell dysfunction. Frequency of DM-2 is higher in certain racial/ethnic groups and has first-degree blood relations, which suggests the operation of unknown genetic susceptibility factors (Banday et al. 2020). The aforementioned data on global and regional DM and DM phenotypes were assembled prior to increasing evidence that infection with SARS-CoV-2 may precipitate new-onset DM, even in COVID-19 patients without predisposing factors for impaired glucose metabolism (Papachristou et al. 2021).

Peripheral neuropathy is the most prevalent chronic complication of DM and may affect 60–70% of patients, of which up to 50% may be asymptomatic (Pop-Busui et al. 2017). While distal symmetrical polyneuropathy (DSPN) accounts for three quarters of DM-related neuropathy, clinical disease may manifest as sensory, motor, and autonomic forms (Kobayashi and Zochodne 2018). Genetic predisposition to the development of painful neuropathy is suspected (Galer et al. 2000). Other forms of DM-related peripheral nervous system (PNS) disease, which includes isolated focal and multifocal cranial or peripheral mononeuropathies, lumbosacral radiculopathy, and proximal motor amyotrophy (Pop-Busui et al. 2017), are not discussed further here.

Diabetic Distal Symmetrical Polyneuropathy

DSPN and its biomarkers, the focus of this chapter, may begin in prediabetes and develop insidiously and progressively if the diabetic state is poorly controlled. Sensory deficits precede motor involvement in a distal, retrograde pattern that reflects degenerative changes in longer before shorter nerve fibers. Symptoms of tingling (hyperesthesia), pain, or loss of sensation first develop in the distal and later in more proximal regions of the lower extremities, subsequently in a similar temporal pattern in the upper extremities, and may even advance to involve the distal regions of sensory nerve fibers supplying the central chest (Kobayashi and Zochodne 2018). The distal predominance of nerve fiber damage is evident in epidermal biopsies that show terminal regions of sensory nerve fibers are more heavily affected than those in more proximal biopsies (Polydefkis et al. 2001). Focal axonal swellings in skin biopsies precede the development of distal nerve fiber loss, are unrelated to painful neuropathy, and represent an early marker of sensory nerve injury in DM-2 (Cheung et al. 2015; Karlsson et al. 2021). While clinical evidence of motor involvement is infrequent, quantitative isokinetic dynamometry reveals muscle weakness at the ankle and knee in both DM-1 and DM-2 neuropathy (Anderson 2012). Motor dysfunction may also result from central nervous system (CNS) changes in DM-1 and DM-2, notably central motor conduction delay that depends on the integrity of corticospinal pathways (Kucera et al. 2005; Muramatsu 2020). Similarly, on the sensory side, electrophysiological studies show central as well as

peripheral somatosensory pathway dysfunction early in DM-1 and DM-2 (Kucera et al. 2005; Rekha et al. 2015). DM-associated CNS and PNS dysfunction is related to disease duration, not the degree of hyperglycemia and metabolic control (Dolu et al. 2003).

DSPN: Central-Peripheral Distal Axonopathy

The term central-peripheral distal axonopathy (CPDA) was introduced to describe nerve fiber length-dependent diseases of the dying-back type (i.e., distal to proximal) that are expressed as symmetrical, distal axonal degeneration occurring concurrently in the peripheral nervous system (PNS) and in selected tracts of the central nervous system (CNS) and which have the clinical stigmata of peripheral neuropathy (Spencer and Schaumburg 1976). In DM, abnormalities of central afferent and efferent pathways revealed by evoked potential studies are probably an expression of CPDA (Comi 1997; Thomas 1999), a distribution of neuropathological damage also seen in the biobreeding *diabetes*-prone rat (Sima and Yagihashi 1985–6). Nineteenth-century anatomical studies of three patients with DM demonstrated distal nerve fiber swelling and degeneration in the dorsal columns, greater in the cervical regions of the gracile tract than in adjacent regions of the cuneate tract and "probably the result of the toxic diabetic blood condition" (Williamson 1894, 1904). Comparable changes in dorsal columns were described in conjunction with loss of both motor and sensory large-diameter myelinated fibers, mostly in the distal regions of peripheral nerves (Greenbaum et al. 1964). Recognition that the longest nerve fibers in peripheral nerves and spinal cord undergo distal retrograde degeneration in a number of toxic conditions led to the understanding of the dying-back process (Cavanagh 1964) and, with systemic γ-diketone intoxication (vide infra), CPDA (Spencer and Schaumburg 1976, 1977). Distal axonopathy produces a clinical picture of polyneuropathy in humans and animals in which sensory and motor disturbances develop in the feet and hands and, with time, progress to the legs and arms (Spencer and Schaumburg 1978). Genetic and toxic models of diabetic neuropathy show the presence of degenerative changes at the distal extremities of dorsal columns and corticospinal tracts (Muramatsu 2020; Schmidt et al. 2000; Tay and Wong 1990), while somatosensory and motor evoked potentials in DSPN reveal abnormalities that are otherwise inapparent early in the disease process and which correlate with the degree of peripheral neuropathy (Dolu et al. 2003; Kucera et al. 2005; Rekha et al. 2015; Sima and Yagihashi (1985–86); Varsik et al. 2001).

The longest sensory axons are those that supply Aβ myelinated nerve fibers innervating mechanoreceptors (Pacinian and Meissner corpuscles), which subserve vibrotactile sensation in the distal lower and upper extremities. Their ability to detect movement depends on the maintenance of delicate intracorpuscular axonal filopod processes that intercalate with modified Schwann cell laminae and which are lost prior to corpuscular denervation (Schaumburg et al. 1974; Spencer and Schaumburg 1976). In the lower extremity, these axons are the peripheral projections of dorsal root ganglion neurons that also maintain centrally projecting axons in the dorsal

spinal columns and which synapse with second-order neurons in the gracile nuclei of the medulla oblongata (Spencer et al. 1973). The central projections of cervical spinal ganglia, which support shorter peripheral axons innervating mechanoreceptors in the upper extremities, run in the spinal cuneate tract and synapse with second-order neurons in the cuneate nucleus. Typically, distal regions of the cuneate tract are spared degenerative changes relative to adjacent regions of the gracile tract, as predicted by CPDA length-dependent differential vulnerability. Nerve fibers of gracile and cuneate neurons decussate in the caudal medulla oblongata as internal arcuate fibers and project to and synapse with third-order neurons in the ventral posterolateral of the thalamus. Axons of ventral posterolateral neurons pass in the internal capsule and terminate in the primary somatosensory cortex (Bajwa and Al Khalili 2021). Given that the central axons of second- and third-order neurons are relatively short, they are thought to be much less susceptible in CPDA; nevertheless, brainstem auditory evoked potential studies reveal early involvement of the central auditory pathway (brainstem-to-midbrain) in DM-2 (Gupta et al. 2013). CNS imaging studies reveal spinal cord atrophy (Eaton et al. 2001; Selvarajah et al. 2006) and a reduction in the volume of cortical gray matter localized to regions involved with somatosensory perception (Selvarajah et al. 2014).

DSPN Etiology and Pathogenesis

DSPN usually develops on a history of long-standing hyperglycemia (major etiologic factor), dyslipidemia, consequent metabolic derangements, and microvessel alterations leading to neuronal inflammation and oxidative stress and mitochondrial dysfunction (Dyck et al. 2011; Sloan et al. 2021). Hyperglycemia promotes irreversible non-enzymatic changes in proteins, lipids, and nucleic acids that form advanced glycation end products (AGEs) in serum and neural tissues (Wada and Yagihashi 2005). Prevention of DSPN and its progression require optimized glycemic control and achievement of cardiometabolic targets (Sloan et al. 2021). While microvascular disease is often but not consistently present in DSPN, it was pointed out long ago that while nerve ischemia can give rise to motor neuropathy, it cannot readily explain the uniform pattern of CPDA in DSPN (Cavanagh 1964; Thomas 1999). While the specific cause of distal axonopathy in diabetes mellitus is unknown, CPDA is shared with a number of distal symmetrical sensory-motor axonal neuropathies, including toxic states that increase the normal endogenous concentration of 2,5-hexanedione (2,5-HD), a physiological γ-diketone that has potential neurotoxicity via the formation of pyrroles with proteins and neuroproteins (Chen et al. 2020; Spencer 2020; Spencer and Chen 2021).

While the molecular cascade arising from hyperglycemia to DSPN is incompletely understood, it is possible to sketch a proposed pathogenesis. Abnormally high concentrations of cellular glucose reduce the rate of degradation of hexokinase-2, which results in elevated glucose metabolism and increased non-aerobic glycolytic energy generation (Irshad et al. 2019); this may explain the elevated physiological nerve resistance to oxygen deprivation during ischemia.

However, increased dependence on glycolysis increases the production of AGE precursors, notably the toxic dicarbonyl compound methylglyoxal (MG, **15** in Fig. 1), which is removed by a cellular (glia>neurons) glyoxylase system that utilizes a non-enzymatic reaction with antioxidant glutathione; this amplifies the opportunity for oxidative damage of cellular macromolecules (Allaman et al. 2015). Immunoperoxidase studies localize AGE deposition not only to axoplasm and Schwann cells of both myelinated and unmyelinated nerve fibers but also to endothelial cells and pericytes, interstitial collagen, and basal laminae of perineural cells; however, these were observed both in non-insulin-dependent diabetic subjects ($n = 5$) and, to a lesser extent, in non-diabetic controls ($n = 5$) (Sugimoto et al. 1997). Although MG injection of normal human skin elicits pain and thermal hyperalgesia reminiscent of DM-associated pain (Düll et al. 2019), results for the association between serum MG concentration and DSPN have been inconsistent (Andersen et al. 2018; Hansen et al. 2015). Nevertheless, the accumulation of AGEs in nerve tissue has been suggested to be a major cause of the onset and progression of neuropathy in humans (Fujita et al. 2021).

While cellular receptors for AGE (RAGE) products counteract the intracellular toxic potential of MG, an immune response is triggered in association with markedly elevated levels of circulating MG in DM-2, which are positively correlated with levels of glycosylated hemoglobin A1c and malondialdehyde (Kong et al. 2014). As the capacity of the dicarbonyl-degrading glyoxalase-1 (GLO-1) system is overwhelmed by increased levels of glycolysis resulting from hyperglycemia, free AGE products such as MG interact with lysine and arginine residues in proteins and neuroproteins. Polymorphism of *GLO-1* appears to affect the onset of DSPN in humans (Fujita et al. 2021). MG detoxification yields 3-hydroxy-2,5-hexanedione (3-HHD, **17**) which, together with the ketone body acetoacetate (AA, **16**), forms pyrrole protein adducts (Salomon et al. 2017), as discussed in detail below. Neurons and their axonal processes have a lower capacity than glia to respond to MG attack, which would render proteins with high lysine content among the most vulnerable. These include the microtubule-associated protein stathmin (15% lysine content), the heavy chain (13–17%) and medium chain (11–15%) of neurofilaments, and the motor proteins kinesin (8–12%), all of which are required for the maintenance of neuronal integrity and, the latter, for the energy-dependent axonal transport of materials synthesized in the nerve cell body (Spencer 2020). Since these materials must be transported over long distances (from nerve cell body to axon terminal), distal regions of the longest PNS and CNS axons will be compromised first, and, with continued metabolic dysfunction, more proximal regions will be similarly affected, while the distal regions of shorter axons also become involved. Distal axons undergo focal swellings at points of slow axonal transport arrest prior to distal nerve fiber degeneration that results in nerve terminal denervation (Spencer and Thomas 1974).

In addition to neuron-specific protein targets of γ-diketones, these preferentially lysine-reactive chemicals also interfere with enzyme-critical functions, notably the pathways involved in energy generation on which neuronal/axonal functions are especially dependent (Ketschek et al. 2021). Indeed, metabolic and toxic CPDA has long been linked with defective energy metabolism in nerve fibers (Spencer et al.

1979), and diminished glycolysis has been directly implicated in DM-2 (Bouché et al. 2004). Under high-glucose conditions, the primary glycolytic intermediate 1,3-bisphosphoglycerate reacts with lysine residues in proteins to form 3-phosphoglyceryl-lysine, which inhibits the activity of glycolytic enzymes (Moellering and Cravatt 2013). MG processing indirectly results in post-translational lactoylation of lysine residues in glycolytic enzymes (Gaffney et al. 2020). Mitochondria are also thought to have an important role in the cellular fate of glucose and the pathogenesis of diabetes (Bouché et al. 2004).

Existing DSPN Biomarkers

Clinical biomarkers of DSPN include increased physiological resistance to ischemia of large- and small-diameter afferent and efferent myelinated and unmyelinated nerve fibers that perhaps indicates greater dependency on anaerobic glycolysis in diabetic nerve (Claus et al. 1990; Nukada 2014; Thomas 1999). Functional biomarkers are abnormal for warm threshold (81.6%), cold threshold (57.9%), vibratory threshold (63.2%), and the amplitude of the sural sensory action potential (49%) that reflects activity in the largest-diameter myelinated nerve fibers (Shun et al. 2004). Vibrotactile sense, which is mediated by dermal Meissner corpuscles (30–50 Hz) and vibration-sensitive (100–400 Hz) Pacinian corpuscles (Spencer and Schaumburg 1973), is diminished early in distal extremities (Dahlin et al. 2008; Lindholm et al. 2019; Peterson et al. 2020; Pourhamidi et al. 2014) and may be useful for the detection of prediabetes sensory nerve damage. The differential detection of vibration by these mechanoreceptors in DSPN has not been tested (Bönhof et al. 2019). Anatomical biomarkers of DSPN include the proximal-distal reducing gradient of epidermal nerve fiber density and fragmented nerve fibers in the dermis (Polydefkis et al. 2001; Shun et al. 2004).

Peripheral neuropathy in DM-2 is associated with inflammation, oxidative stress, and mitochondrial dysfunction (Román-Pintos et al. 2016). Thus, molecular markers of diabetic neuropathy include those associated with inflammation (MCP-1, VEGF, TRPV1, NF-κB), oxidation (adiponectin, NFE2L2), enzyme activity (NADPH, ceruloplasmin, HO-1, DPP-4, PARP-α), and other functions (SIRT1, caveolin 1, MALAT1, and miRNAs 199a-3p, 146, and 499a) (Adki and Kulkarni 2020; Xourgia et al. 2018). Disease progression is said to be associated with increased levels of IL-6, IL-1, TNF-α, and TGF-β (Jin and Park 2018). Glycated hemoglobin (HbA1c) has been proposed as a readily assayable biomarker for diabetic foot peripheral neuropathy (Casadei et al. 2021). Elevated plasma levels of pro-inflammatory factors predicted the onset of DSPN in DM-affected residents of Europe (TNF-α and IL-6) and China (TNF-α and ICAM-1) (Herder et al. 2017; Zheng et al. 2020), while sICAM-1 and IL-1RA were positively associated with progression of DSPN in Europeans (Herder et al. 2017). While systemic vascular inflammation is well known in DM, an etiologic role in DSPN has not been demonstrated. Markers of nerve fiber damage in a rodent model of diabetic neuropathy include downregulated structural proteins (neurofilament, tubulins) and nerve

growth factor, upregulation of nitrotyrosine, increase in intracellular calcium, and overactivation of poly(ADP-ribose) polymerase (Shaikh and Somani 2010).

The subject of serum molecular biomarkers in diabetic neuropathy has been reviewed recently in the context of regenerative medicine (Fujita et al. 2021). Four groups of biomarkers were identified, including AGE-related molecules potentially related to disease etiology, those involved in the progression of inflammation (e.g., Toll-like receptors, TNF-α, miR-146a, adiponectin), molecules associated with nerve fiber damage (neuron-specific enolase, semaphorin), and those associated with nerve protection (nerve growth factor and HSP27). Since the first group of molecules is addressed here in relation to DSPN etiology, the interested reader is referred to this excellent review for information on the three other groups of potential serum biomarkers of DSPN.

Protein Pyrrole Adducts

Our recent studies of diabetic neuropathy have shown a potential relationship with the formation of 2,5-dimethylpyrroles detectable in urine (Chen et al. 2020). Pyrroles form in non-enzymatic reactions with proteins (shown in Fig. 1) that occur in various diseases, of which the best studied is *n*-hexane (**1**) or methyl *n*-butyl ketone (2-hexanone) neuropathy, where the active neurotoxic metabolite is the γ-diketone 2,5-HD (**2**). Repeated exposure to γ-diketones leads to sensory abnormalities and motor dysfunction in distal extremities in association with the formation of giant axonal swellings filled with 10 nm neurofilament, localized secondary demyelination, and distal axonal degeneration of large-diameter myelinated nerve fibers. 2,5-HD undergoes Paal-Knorr synthesis (shown in Fig. 1a) to form a pyrrole: the reaction starts when one electrophilic carbonyl of 2,5-HD attacks a primary amine, especially the epsilon amino group of lysine residues in proteins, to form a hemiaminal (**3**), which results in an imine (**4**) that isomerizes to an enamine (**5**), and then attacks the other unreacted carbonyl and cyclizes to form a dimethylpyrrole (**6**) with subsequent dehydration (Graham et al. 1982). Paal-Knorr condensation of γ-diketones is a first-order reaction, and its rate-limiting step involves cyclization of the hemiaminal (**3**) (Amarnath et al. 1991).

Pyrrole generation as the initial molecular event in the pathogenesis of *n*-hexane neurotoxicity is supported by studies of structure-activity relationships among diketones (shown in Table 1): (a) γ-diketones that form pyrrole adducts show neurotoxicity, while no neurotoxicity is observed for α- and β-diketones that react with primary amines but do not form pyrrole adducts (Spencer et al. 1978; DeCaprio et al. 1982); (b) methyl substitution at carbons C3 and C4 (to form 3,4-dimethyl-2,5-hexanedione, DMHD, **9** or **10**) accelerates protein pyrrole formation, reduces activation energy, and is 20–30 times more potent than 2,5-HD in triggering a neurofilamentous axonal neuropathy (Anthony et al. 1983a, b, c; Sanz et al. 1995; Xu et al. 2001); (c) di-methyl substitution of carbon C3 (to form 3,3-dimethyl-2,5-hexanedione, **11**) poses steric hindrance to pyrrole adduct formation and causes slight changes of axon caliber but no clinical evidence of neurotoxicity (Sayre et al.

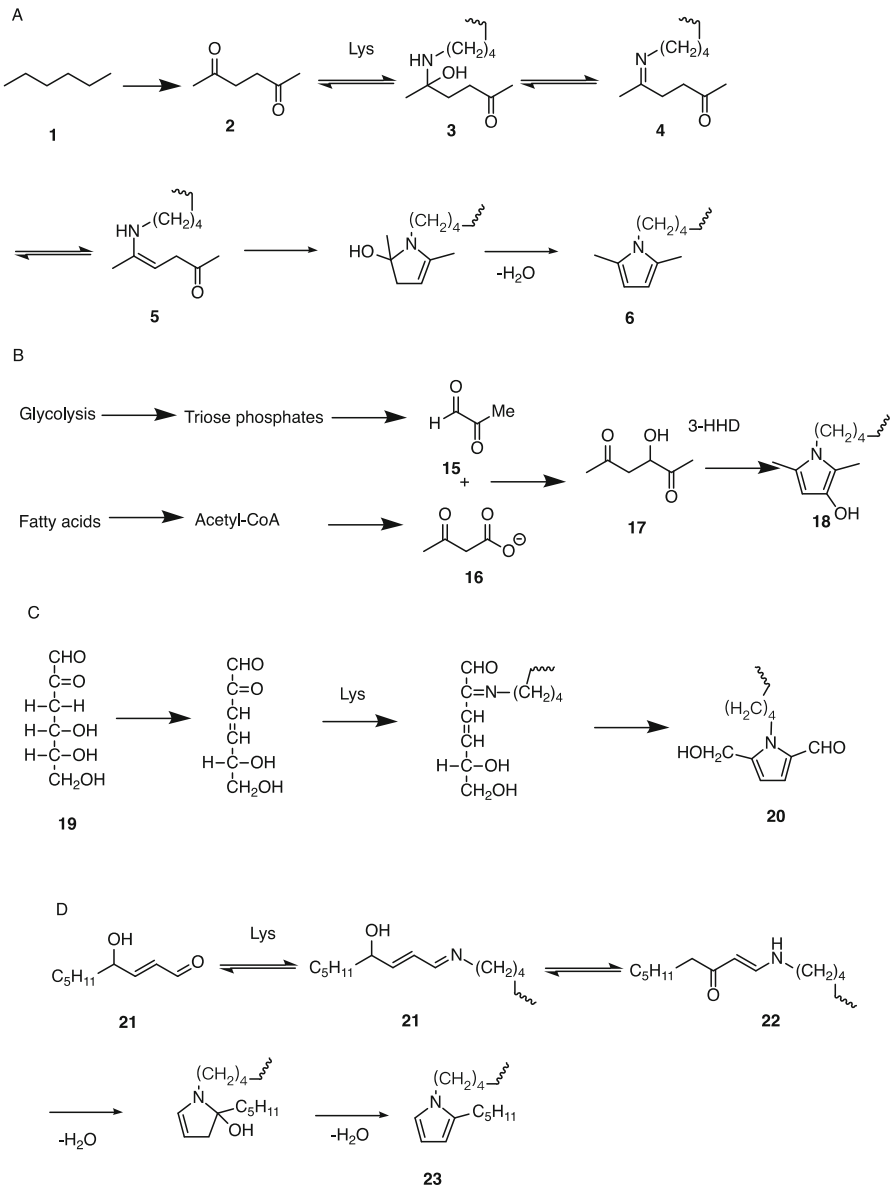
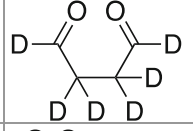
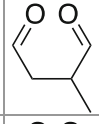
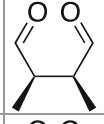
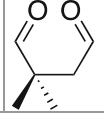


Fig. 1 Reactions that form protein pyrrole adducts

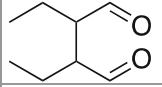
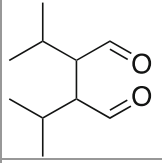
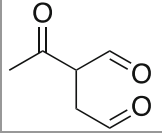
1986); (d) di-methyl substitution at C3 and C4 (DMHD) results in a more rapid pyrrole formation than with 2,5-HD, but ethyl (3,4-diethyl-2,5-hexanedione, DEHD, **12**) and isopropyl (3,4-diisopropyl-2,5-hexanedione, DiPHD, **14**) substitution at C3 and C4 position slows pyrrole formation relative to 2,5-HD (Szakal-Quin et al. 1986); neither axonal swelling nor Wallerian-type degeneration is observed in

Table 1 Structure toxicity relationship of aliphatic γ-diketones

Name (Abbreviation, number)	Structure	Capacity in forming pyrrole	Capacity in auto-oxidation/cross-linking	Neurotoxicity	Reference
2,5-Hexanedione (2,5-HD, **2**)		1	1	1	(DeCaprio et al. 1982; Graham et al. 1982)
Perdeuterio[D10]-2,5-HD, **7**		<1	–	<1	(DeCaprio et al. 1988)
3-Methyl-2,5-hexanedione (3MHD, **8**)		>1	>1	>1	(Sayre et al. 1986; Xu et al. 2001)
d,l-3,4-Dimethyl-2,5-hexanedione (d,l-DMHD, **9**)		> meso-DMHD	> meso-DMHD	> meso-DMHD	(Rosenberg et al. 1987b; Szakal-Quin et al. 1986)
meso-3,4-Dimethyl-2,5-hexanedione (meso-DMHD, **10**)		>MHD	>MHD	>MHD	(Anthony et al. 1983b; Sayre et al. 1986)
3,3-Dimethy-2,5-hexanedione (3,3-DMHD, **11**)		0	–	0	(Sayre et al. 1986)

(continued)

Table 1 (continued)

Name (Abbreviation, number)	Structure	Capacity in forming pyrrole	Capacity in auto-oxidation/cross-linking	Neurotoxicity	Reference
3,4-Diethylhexane-2,5-dione (3,4-DEHD, **12**)		<1	>1	<1	(Genter et al. 1987)
3,4-Diisopropyl-2,5-hexanedione (DiPHD, **13**)		<3,4-DEHD	1 < DiPHD <3,4-DEHD	<1	(Genter et al. 1987)
3-Acetyl-2,5-hexanedione (AcHD, **14**)		>1	~0	0	(Pyle et al. 1992)

animals treated with DEHD or DiPHD (Genter et al. 1987); 5) *d*,*l*-diastereomeric forms of γ-diketones form pyrroles 4–40 times faster than meso-diastereomeric forms (Szakal-Quin et al. 1986), and *d,l*-DMHD (**9**) speeds hindlimb paralysis and shows more distal axon swelling than meso-DMHD (**10**) (Rosenberg et al. 1987b); and 6) serum levels of pyrrole adducts in rats treated with 2,5-HD are 2–3 times higher than those in animals receiving equivalent doses of perdeuterio-2,5-HD ([D10]-2,5-HD, **7**), in parallel with the higher neurotoxic potency of 2,5-HD compared with [D10]-2,5-HD (DeCaprio et al. 1988). The protein pyrrole adducts are found in the urine, globin, hair, serum, liver, kidney, brain, and purified myelin, and pyrrole formation is dose- and time-dependent in animals treated with *n*-hexane or 2,5-HD (DeCaprio et al. 1983; Yin et al. 2013, 2014a; Li et al. 2018).

Low levels of urinary 2,5-HD (mean: 0.35–1.47 mg/L) and pyrrole adducts (median: 0.91–7.4 μM) are found in healthy populations without occupational exposure to *n*-hexane or 2-hexanol, as reviewed by Spencer and Chen (2021). The origin of endogenous 2,5-HD is unclear, but it may result from lipid oxidation or endogenous *n*-hexane, which is also a product of lipid oxidation. 3-Hydroxy-2,5-hexanedione (3-HHD, **17**), another type of endogenous γ-diketone, is generated from a non-enzymatic detoxification reaction (shown in Fig. 1b) of the highly reactive glycating agent MG (**15**); additionally, the ketone body acetoacetate (AA, **16**) is also able to form pyrrole protein adducts (Salomon et al. 2017). 3-HHD is detectable in ketototic patients with levels ranging from 10 to 40 nM; this is two to three orders of magnitude lower than the concentration of urinary 2,5-HD in healthy populations, which ranges from 0.35 to 1.47 mg/L (3.5–10 μM). Unlike 2,5-HD, 3-HHD may further oxidize to triketones that could be even more potent glycating species (Johannsen 2021, personal communication).

Non-enzymatic glycosylation occurs between reducing sugars and amino residues of proteins, giving rise to AGEs, many of which have a pyrrole structure (Fig. 1c). For example, 3-deoxyglucosone (**19**) reacts with neopentylamine or bovine serum albumin under physiological conditions of pH and temperature to form 5-hyroxymethylpyrrole-2-carbadehyde (pyrraline) (**20**) (Njoroge et al. 1987; Hayase et al. 1989). 4-Hydroxy-2-nonenal (4-HNE, **21**), an α,β-unsaturated hydroxyalkenal generated in the peroxidation of n-6 and n-3 fatty acids (Van Kuijk et al. 1990), is able to form protein pyrrole adducts (**23**) via an imine (**21**) that maintains equilibrium with an enamine (**22**) (Sayre et al. 1993; Vazdar et al. 2017). This reaction, which yields 14–23% of pyrrole, seems to be a side reaction (Fig. 1d) via Michael addition that finally generates 4-HNE-derived 2-pentylfuran (Sayre et al. 1993) or cyclic hemiacetal (Amarnath et al. 1998); albeit, the pyrrole adduct is more thermodynamically stable than either the Michael adduct or hemiacetal (Skulj et al. 2019).

Cross-Linking of γ-Diketone-Derived Pyrroles

Many γ-diketone-derived pyrroles do not absorb within the visible spectrum, or they are colorless (Graham et al. 1982); however, prolonged incubation of γ-diketones with primary amines or proteins generates a chromophore, the color of which

depends on the structure of the γ-diketone. For example, amines react with 2,5-HD to form a yellow chromophore, with DMHD a reddish-brown chromophore, with DEHD an orange chromophore, and with DiPHD a pink chromophore (Genter et al. 1987). Rats treated with aliphatic γ-diketones develop an orange discoloration, while animals given the aromatic γ-diketone 1,2-diacetylbenzene exhibit a blue pigmentation of skin and internal organs, including the brain, spinal cord, and nerves (Kim et al. 2001). Chromophore generation is attributed to auto-oxidation and, thereafter, cross-linking of pyrrole adducts, both of which are considered to be required for γ-diketone neurotoxicity for the following reasons: (a) air-exposed mixtures of 2,5-HD and bovine serum albumin develop the chromophore with the reduction of pyrrole adduct monomers, while under inert nitrogen, the same mixture neither generates a chromophore nor reduces the pyrrole adduct monomers (DeCaprio 1986); (b) addition of free-radical initiators accelerates the formation of the chromophore, while antioxidants inhibit both chromophore formation and cross-linking (DeCaprio 1986; Zhu et al. 1994); (c) hyperbaric oxygen treatment of rats treated with 2,5-HD significantly speeds the development of hindlimb paralysis (Rosenberg et al. 1987a); (d) the *d,l*- diastereomeric form of DMHD has a greater in vitro cross-linking rate than meso-DMHD, and both exceed the corresponding rate for 2,5-HD, which parallels the order of their relative neurotoxic potency in vivo (i.e., *d,l*-DMHD > meso-DMHD > 2,5HD) (Genter et al. 1987; Rosenberg et al. 1987b); and (e) 3-acetyl-2,5-hexanedione (ACHD, **14**), a γ-diketone that forms protein pyrrole adducts but resists auto-oxidation and therefore has a very low cross-linking rate, lacks the ability to induce hindlimb paralysis in treated rodents (Pyle et al. 1992).

Pyrrolecarboxyaldehydes (**24**) together with methylene-bridged dimers (**25**) of pyrroles are found in the reaction mixture of γ-diketone plus a model amine, which suggests the methyl group of the dimethylpyrrole adduct of 2,5-HD is first converted to a CHO group and then dimerizes during the auto-oxidation (Xu et al. 2001; Zhu et al. 1993). A novel type of pyrrole-pyrrole dimer with an extended π-bond area and the reaction to generate the structure has been proposed (shown in Fig. 2a) to explain the chromogenic and associated neurotoxic properties of γ-diketones (Zhan et al. 2004). This pyrrole-pyrrole bridging mediates an intermolecular cross-linking pathway of many types of proteins, including serum albumin (DeCaprio 1986), axonal cytoskeletal proteins (DeCaprio et al. 1988), ribonuclease (Zhu et al. 1995), and the spectrin of erythrocyte ghosts (Pyle et al. 1992), while this pyrrole-pyrrole bridging is compromised by the conjugation of free thiols present in proteins, glutathione, and other low-molecular-mass thiols (Zhu et al. 1995; Torres et al. 2014).

The triplet proteins of 10 nm neurofilaments were hypothesized to be the primary axonal molecular target of γ-diketones, as the covalent cross-linking of neurofilaments leads to the obstruction of anterograde transport, large neurofilament-filled swellings in distal regions of elongate nerve fibers that undergo degeneration (Genter et al. 1987). However, this hypothesis was challenged by the observation that neurofilament-deficient animal models also developed γ-diketone-induced distal axonal degeneration (Stone et al. 1999, 2001). A number of other

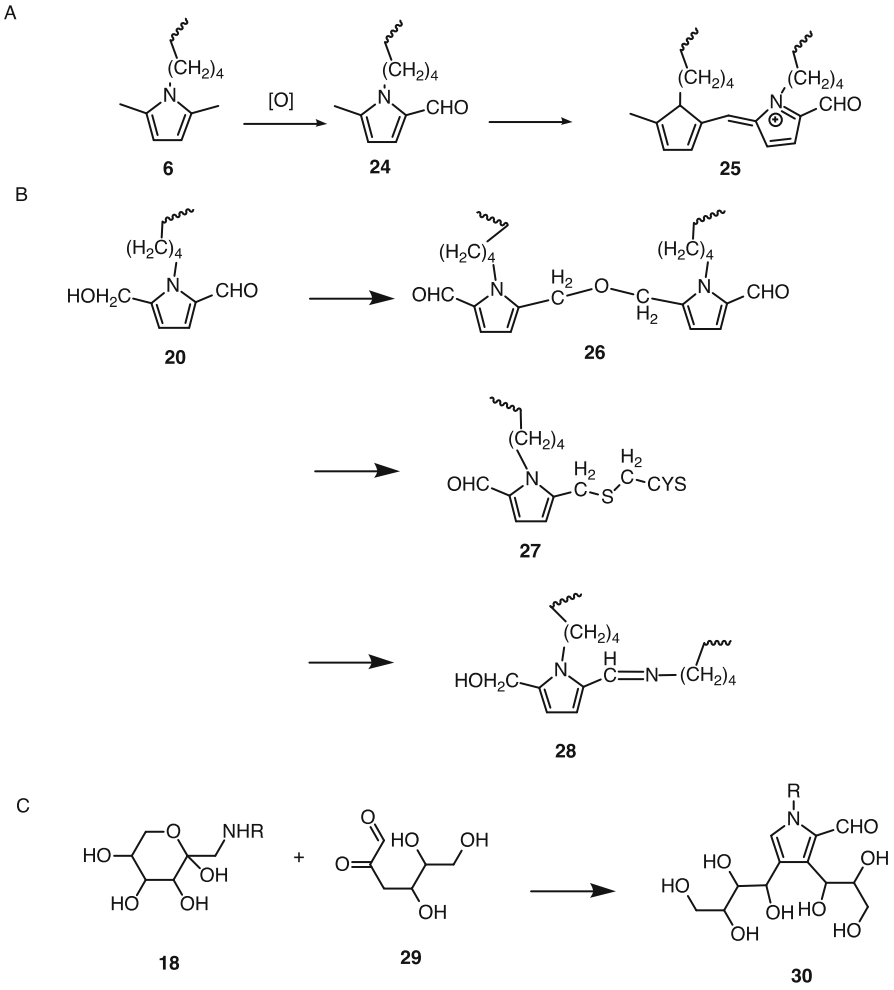


Fig. 2 Reactions that form protein cross-linking

axonal proteins is involved in the induction of γ-diketone neuropathy (Spencer 2020).

Noted above is that healthy subjects with no known exposure to exogenous *n*-hexane or 2-hexanone, or indeed any other known source of γ-diketone, have detectable levels of apparently endogenously generated serum and/or urine 2-hexanone, 3-heptanone, and 2,5-HD, together with 2-butanone, which potentiates the neurotoxic potential of *n*-hexane (Zlatkis et al. 1980; Spencer and Chen 2021). While serum levels of 2,5-HD in normal subjects are low, it is reasonable to expect the formation and cross-linking of protein pyrrole adducts that, in the long term, could potentially lead to axonopathy. Indeed, this possibility has been explored in

relation to the distal neuropathies associated with diabetes mellitus (Spencer and Chen 2021; Chen et al. 2021) (vide infra).

Cross-Linking of Protein Pyrrole Adduct: Links with DSPN

Protein cross-linking is the molecular process of joining two or more molecules by a covalent bond. Many chemical structures other than γ-diketones are able to cross-link proteins and thus are neurotoxic candidates. For example, 4-hydroxy-2-nonenal, a reactive aldehyde and product of lipid peroxidation, is reported to form a pyrrole adduct with apolipoprotein and tau (Montine et al. 1997a, b). The adduct was thought to be formed mainly through Michael addition (Montine et al. 1996a, b) until an antibody specific to the 4-HNE-derived pyrrole adduct was developed and used to visualize the distribution of protein cross-links in neurofibrillary tangles in the hippocampus and temporal cortex of APOE4-homozygous patients with Alzheimer disease (Montine et al. 1997a, b, 1998). Various neuropsychiatric (schizophrenia, depression) and neurologic disorders (amyotrophic lateral sclerosis and Alzheimer, Huntington, and Parkinson diseases) have been associated with elevated HNE levels in the plasma, cerebrospinal fluid, or brain tissue (Romano et al. 2017; Di Domenico et al. 2017). 4-HNE is also reported to be elevated in the sera of diabetic patients (Lou et al. 2020), in the dorsal ganglia root cells of diabetic mice (Zherebitskaya et al. 2009), in zebrafish larvae with impaired glucose homeostasis (Lou et al. 2020), and in the dorsal root ganglia of streptozotocin-induced diabetic rats (Lupachyk et al. 2011). 4-HNE induces reactive oxygen species, forms pyrrole adducts with mitochondrial proteins, and impairs axonal outgrowth from rat sensory neurons in vitro (Akude et al. 2010; Zherebitskaya et al. 2009). Additionally, serum 4-HNE levels are increased in type 2 diabetic patients and correlate with disease progression (Toyokuni et al. 2000). In sum, 4-HNE is an etiologic candidate for the induction of diabetic sensory neuropathy (Akude et al. 2010).

Pyrraline (epsilon 2-(formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine) is an advanced Maillard reaction product and AGE derived from the reaction of glucose with protein lysines (Nagaraj et al. 1996). Pyrraline's cross-links to proteins are highly diversified, possibly due to the substituents of the pyrrole ring. Pyrraline cross-links to protein (shown in Fig. 2b) can form by the substitution of hydroxy groups in the α-position of pyrraline to another pyrraline (Nagaraj et al. 1996) or cysteine residue (Klein et al. 1992) or by condensation between the pyrrole aldehyde and primary amine of lysine to form pyrrole carbimine derivatives (**28**) (Nissl et al. 1995). Pyrraline is detected in plasma proteins, connective tissue, and optic nerve head of patients with elderly DM patients to a far greater degree than diabetes-free controls (Amano et al. 2001). In spite of the strong associations between AGE and DSPN (Misur et al. 2004; Wan et al. 2019), AGE treatment affected endoneurial vascular function but failed to induce significant differences from controls in mean total fascicular area, myelinated fiber density, myelinated fiber number, and mean myelinated fiber size (Nishizawa et al. 2010). AGEs are highly diverse in structure, and, among other AGEs, pyrraline failed to react immunologically with an anti-AGE

antibody whose domain epitope forms from the reaction of glucose with proteins under native conditions (Makita et al. 1992). Besides, the levels of pyrraline were discrepant among reports: while two studies reported elevated plasma pyrraline (mean: 21.6 pmole/mg vs. 12.8 pmole/mg) and serum albumin (mean: 43 pmole/mg vs. 27 pmole/mg) in diabetic subjects (Hayase et al. 1989; Portero-Otin et al. 1995), a third found no detectable pyrraline in either diabetic or non-diabetic samples (Smith et al. 1993). In sum, more evidence is needed to support the proposed link between pyrraline and DSPN.

Another type of pyrrole-based AGE, namely, alkyl formylglycosyl pyrrole (AFGP, **30**), is generated from 3-deoxyglucosone (**18**) and 6-aminohexanoic acid (**29**), an Amadori product of glucose and lysine (Farmar et al. 1988). Though the formation of AFGP is the product of cross-linking between two molecules of reducing sugar and one molecule of lysine, there are limited reports of AFGP in vivo (Ahmed 2005). Whether pyrrole-structured AGEs can induce distal axonopathy, and the possible role of pyrrole-structured AGEs in DSPN, requires further assessment.

Detection of Pyrrole Adducts

The most common method for the determination of pyrrole protein adducts is spectrophotometry using Ehrlich's reagent, the active ingredient of which is *p*-dimethylaminobenzaldehyde (DMAB). DMAB reacts with pyrrole to form a pyrrolium adduct (Alexander and Butler 1976) that forms a pink chromophore ($\lambda max = 526$ nm) for quantification. Ehrlich's reagent has been exploited to measure tryptophan ($\lambda max = 640$ nm) (Nixon 1973) and urea ($\lambda max = 420$ nm) (Yatzidis et al. 1964). However, the presence of porphobilinogen ($\lambda max = 555$ nm) can interfere with the measurement of pyrrole protein adducts) (Roper 2012). For this reason, an individual with a positive urobilinogen test should be excluded from pyrrole protein assays. Spectrophotometry assay requires only small amounts of sample, and the analytic method is facile. The assay is able to monitor the dynamics of protein pyrrole adducts and to measure diversity among samples. Using this method, Yin and colleagues measured and monitored the level of pyrrole adducts in the serum, urine, liver, kidney, brain, and sciatic nerve of rats treated with *n*-hexane by gavage or by intraperitoneal 2,5-HD (Yin et al. 2013, 2014b). The level of protein pyrrole adducts in serum correlated well with the cumulative dose of 2,5-HD (Yin et al. 2014a, b).

Formulation of Ehrlich's reagent can be modified to stain SDS-PAGE gels for the exploration of the protein targets of γ-diketones or other reagents that generate pyrrole structures (DeCaprio et al. 1982; Campbell et al. 2010). However, due to low sensitivity and high cross-reactivity among different pyrrole adducts, the results obtained from spectrophotometry should be regarded as preliminary data that require confirmation.

Immunological assay using antibodies to pyrrole adducts has been used to detect the presence or distribution of protein pyrrole adducts in various samples. Compared

with DMAB, the antibodies to pyrrole adducts are much more specific: for example, pyrraline antibodies have low (<0.03%) detectable cross-reactivity with other non pyrraline-derived pyrrole and furan analogues (Hayase et al. 1989). Antibody pyrrole adducts derived from pyrraline (Hayase et al. 1989) and 4-HNE (Montine et al. 1997b) have been developed and used in the aforementioned immunohistochemistry studies. To the best of our knowledge, few studies have exploited specific γ-diketone antibodies to explore the targeted proteins of γ-diketones that underpin the molecular basis for γ-diketone CPDA.

Mass spectrometry and nuclear magnetic resonance (NMR) are used to confirm the presence and identity of protein pyrrole adducts because of the high sensitivity and specificity of these two methods. Both methods have been used intensively to study the thermodynamics and kinetics of pyrrole adduction, for example, to determine the structure (Amarnath et al. 1991) and the stereochemistry (Szakal-Quin et al. 1986) of the reaction intermediates. However, delicate instruments and professionals are required, and finding the fingerprints of pyrrole adducts from massive spectra generated by these methods is also difficult. Mass spectrometry is preferred in recent studies in the measurement of clinical samples (Salomon et al. 2017) since NMR is limited to "clean" agents and simplified reactions.

Applications to Peripheral Neuropathy

Occupational *n*-Hexane/2-Hexanone Neuropathy

Due to the short half-life of 2,5-HD in humans (8.3–13.3 h) (Filser et al. 1996), the urinary 2,5-HD level has limited value in the diagnosis of *n*-hexane and 2-hexanone polyneuropathy. Nevertheless, the aforementioned studies of animals treated with *n*-hexane or 2,5-HD reveal good correlations with pyrrole adduct levels and peripheral neuropathy. It is thus reasonable to consider protein pyrrole adducts as an alternative biomarker to assess human exposure to *n*-hexane or 2-hexanone. Ichihara and colleagues were the first to report concentrations of protein pyrrole adducts in the blood of subjects with occupational exposure to *n*-hexane (Ichihara et al. 2019). Whole blood for the measurement of plasma and globin pyrrole adducts was collected from 11 *n*-hexane-exposed workers and 4 non-exposed healthy controls, and the level of protein pyrrole adducts was evaluated with Ehrlich's reagent. To exclude absorbance attributed to non-pyrrolated proteins, a distinct method of calculation was employed: three wavelength absorbances (510 nm, 530 nm, and 550 nm) were measured, and then the mean value of the absorbance at 510 nm and 550 nm was subtracted from that at 530 nm. The level of pyrroles was calculated based on the difference values between absorbances at 530 nm and the mean absorbance at 550 nm and 510 nm, using the formula (A530 − (A510 + A550)/2), and then the difference values were converted to concentrations of pyrrole adducts, referring to a standard curve prepared with different concentrations of 1-(2-hydroxyethyl)-2,5-dimethylpyrrole (HeDMP) with Ehrlich's reagent. Although this approach was claimed to be reproducible

and to avoid interference from other non-pyrrolated proteins that have absorbances in the region of interest, it was based on the hypothesis that endogenous 2,5-HD and 2,5-HD-derived pyrrole are undetectable. Therefore, the level of 2,5-HD-derived pyrroles among *n*-hexane-exposed workers might also have been underestimated. Since the biological lifetime of globin (120 days) is much longer than that of plasma protein (20 days), the globin pyrrole adduct may represent a better biological indicator of chronic exposure to *n*-hexane.

Diabetic Polyneuropathy

Given that 2,5-HD appears to be a normal endogenous metabolite (vide supra), albeit of unknown origin and fate in humans, and both *n*-hexane neuropathy and DSPN have a common distribution of neuropathology (i.e., CPDA), we hypothesized that DSPN may arise from an elevation of endogenous γ-diketone, the derived pyrrole adducts of which could be detected in urine (Spencer and Chen 2021). This hypothesis was tested by measuring the urinary concentration of γ-diketone pyrroles in age-matched and gender-matched elderly (60–84 years) persons with ($n = 267$) or without ($n = 267$) indicators of DM based in a community population in China (Chen et al. 2020). Compared with healthy controls, those with DM had significantly higher levels of fasting blood glucose, glycated hemoglobin A1c, urinary ketone bodies, and urinary γ-diketone pyrroles. The median concentration of urinary pyrrole adducts determined spectrophotometrically was significantly higher ($p < 0.0001$) in the diabetic group (7.5 μM or 0.99 mg/g urine creatinine) than in healthy subjects (5.9 μM or 0.70 mg/g urine creatinine). The median urinary pyrrole adduct level (7.4 μM) was at the same order of magnitude but higher than the total urinary level 2,5-HD (0.159 mg/L or 1.5 μM) for the Chinese population, as determined by mass spectrometry (Pan et al. 2016). In sum, while urinary γ-diketone pyrroles are elevated in DM, further studies are required to determine whether this observation correlates with DSPN.

Conclusions

Diabetic dysmetabolism includes increased generation and excretion of neuropathy-associated γ-diketone pyrroles. These findings form the foundation for ongoing studies designed to test whether γ-diketone pyrrole concentration correlates with quantitative sensory (vibration and temperature) and electrodiagnostic testing results in DM-2. Preliminary observations suggest that the level of γ-diketone-pyrroles in urine can be used to distinguish between diabetic and healthy human subjects with no occupational or other exposure to γ-diketone precursors (i.e., *n*-hexane, 2-hexanone), but further studies are needed to determine whether serum levels of both 2,5-HD and associated γ-diketone-pyrroles are similarly elevated in DM-2 and correlate with DSPN. Many other types of protein pyrroles are present in human serum and raised in DM, but, other than 4-HNE, evidence in support of an etiological

role in DSPN is lacking. Genetic risk factors for DSPN may exist, among which are enzymes involved in the removal of reactive carbonyls such as MG (i.e., GLO-1).

Mini-Dictionary of Terms

- AGE: Advanced glycation end products resulting from the chemical transformation of amine-containing compounds (proteins, lipids, nucleotides) by reducing sugars to form Maillard products, such as the potent dicarbonyl glycating agent methylglyoxal (reduced derivative of pyruvate), which reacts with lysine and arginine residues of proteins. RAGE refers to cellular receptors for AGEs.
- Biomarker: A measurable physiological state, anatomical structure, or biological substance, the presence of which in an organism is indicative of a metabolic process, environmental exposure, or disease state.
- Cross-linking: The process of chemically joining two or more molecules by a covalent bond.
- CPDA: Central-peripheral distal axonopathy, a primary disease of elongate central and peripheral axons that describes the nerve fiber pathology underlying many types of metabolic and toxic neuropathies, including DSPN.
- Distal axonopathy: Shorter form of CPDA.
- DM-1. Type 1 diabetes mellitus, an insulin-dependent disorder of usual juvenile onset that results from an autoimmune assault on pancreatic β-cells.
- DM-2. Type 2 diabetes mellitus, a non-autoimmune form of diabetes characterized by insulin resistance and pancreatic β-cell dysfunction.
- DSPN: Diabetic sensory/sensorimotor polyneuropathy that occurs in DM-1 and DM-2 in the form of a stocking-and-glove sensory abnormality that may later involve motor dysfunction in a similar distribution.
- Prediabetes: A pathophysiological state characterized by slightly elevated levels of blood glucose that indicate a risk of progression to DM-2.
- Pyrrole: Any of a class of organic compounds with a five-membered ring composed of four carbon atoms and one nitrogen atom.

Key Facts

- Diabetes mellitus (DM) is a chronic metabolic disease of people of all ages and may appear during pregnancy. The disease was estimated to affect almost one half billion people worldwide in 2019, with projected increases of ~25% by 2030 and ~ 50% by 2045. DM prevalence increases with age, as illustrated by the doubling of disease prevalence among South Korean adults aged >65 years (28%) vs. those aged >30 years (13.8%). Conditions associated with diabetes include obesity, hypertension, elevated levels of blood cholesterol, and peripheral neuropathy. The global estimated age-standardized incidence rate for both DM type 1 (DM-1) and DM type 2 (DM-2) grew between 1990 and 2017, with the largest increase in high-income countries.

- The causes of DM are not understood. DM-1, which accounts for 5–10% of DM cases, is an autoimmune, insulin-dependent disorder usually of juvenile onset but may also occur in later life. DM-2, a non-autoimmune form of DM that constitutes ~90–95% of DM cases, whether or not they are overweight or obese, is characterized by insulin resistance and dysfunction of the beta cells in the pancreas. The frequency of DM-2 is higher in certain racial/ethnic groups and first-degree blood relations. Infection with SARS-CoV-2 may precipitate new-onset DM, even in COVID-19 patients without predisposing factors for impaired glucose metabolism.
- Diabetic Neuropathy. The most prevalent form of diabetic neuropathy is associated with disordered sensation (with or without pain) in the feet and hands. While widely considered a disease of the ends of peripheral nerves, concurrent and often silent involvement of the central nervous system has been recognized recently, although changes in the spinal cord were first described in diabetic patients >125 years ago. The cause of diabetic neuropathy is not clearly understood but primarily evolves from elevated levels of blood sugar that promote formation of toxic molecules that can damage critical nerve proteins. Continuous control of blood sugar levels can stem the advance of diabetic neuropathy.

Summary Points

- The γ-diketone 2,5-HD is a normal physiological metabolite, but neurotoxic levels resulting from exposure to *n*-hexane and 2-hexanone cause central-peripheral distal axonopathy comparable in distribution to that of DSPN.
- γ-Diketones form pyrroles with proteins and neuroproteins, the products of which undergo oxidation and cross-linking of proteins.
- Compared with healthy Chinese controls, age-matched subjects with diabetes mellitus have significantly higher levels of urinary γ-diketone pyrroles.
- Whether diabetic polyneuropathy is triggered by pathophysiological levels of pyrrole-forming endogenous 2,5-HD and/or excessive glycation end products such as 4-HNE requires further study.
- Clinical biomarkers of DSPN include increased physiological resistance to ischemia of large- and small-diameter afferent and efferent myelinated and unmyelinated nerve fibers.
- Functional biomarkers of DSPN include abnormal warm and cold thresholds, abnormal mechanoreceptor thresholds, and an abnormal amplitude of the sural sensory action potential.
- Anatomical biomarkers of DSPN include the proximal-distal reducing gradient of epidermal nerve fiber density and fragmented nerve fibers in the dermis.
- Serum biomarkers associated with DM include AGE-related molecules potentially related to disease etiology, notably 4-HNE, as well as those involved with the progression of inflammation, nerve fiber damage, and protection.

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Biomarkers in Gestational Diabetes

49

Peak Glucose Monitoring at Different Mealtimes

Aykan Yucel and Betul Yakistiran

Contents

Application to Prognosis, Other Diseases or Conditions 1022
Application to Prognosis 1022
Application to Other Diseases or Conditions 1022
Introduction 1022
Noncontinuous Versus Continuous Glucose Monitoring: 1024
Postprandial Versus Preprandial Glucose Monitoring: 1025
Updated Guidelines 1027
Conclusion 1028
Mini Dictionary of Terms 1028
Key Facts 1028
Summary Points 1029
References 1029

Abstract

Gestational diabetes mellitus is defined as new-onset hyperglycemia and carbohydrate intolerance during the second and third trimester of pregnancy without a history of diabetes. It may complicate the pregnancy process and adverse perinatal and maternal pregnancy outcomes may occur. The optimal glycemic control is so important to improve pregnancy outcomes. This chapter describes the techniques of blood glucose monitoring, optimal timing for sampling, adverse maternal and fetal outcomes, and the content of updated evidence-based guidelines for gestational diabetes mellitus.

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Keywords

Gestational diabetes mellitus · Blood glucose monitorization · Postprandial glucose monitoring · Self-glucose monitoring · Macrosomia

Abbreviations

ACOG	The American College of Obstetricians and Gynecologists
ADA	American Diabetes Association
CGM	Continuous blood glucose monitoring
GDM	Gestational diabetes mellitus
LGA	Large-for-gestational age
NCGM	Non-continuous glucose monitoring
NICE	National Institute for Health and Care Excellence
OGTT	Oral glucose tolerance test

Application to Prognosis, Other Diseases or Conditions

Application to Prognosis

In this chapter, blood glucose monitoring technique and protocols have been reviewed. Finger stick blood glucose monitoring and postprandial 1-h or 2-h blood glucose measurements are assessed in general clinical practice. As a new protocol, the authors focused on postprandial 90th min glucose values that have been reported in previous studies (Sever et al. 2021). When the 90th-min blood glucose limit value was determined as 130 mg/dl, there was no significant relationship between high blood glucose levels and complications except neonatal intensive care unit requirement. There are different follow-up protocols in conflict with each other. However, either postprandial 1-h or 2-h glucose values for follow-up are recommended by ADA and ACOG.

Application to Other Diseases or Conditions

Assessment of 90th-min blood glucose levels may be clinically useful for diabetes mellitus type 1 and 2. To the best of our knowledge, there has been no study that compares the clinical outcomes and long-term prognosis of postprandial 90th-min blood glucose level in the patients who are complicated by diabetes mellitus and the other medical disorders with insulin resistance.

Introduction

Gestational diabetes mellitus (GDM) is defined as new-onset hyperglycemia and carbohydrate intolerance during pregnancy without a history of diabetes (ACOG 2018). GDM accounts for 90% of hyperglycemia that is diagnosed in pregnancy and

from day to day its prevalence has increased to above 15% of all around the world (Zhu and Zhang 2016). The diagnosis of GDM is based on the results of a 75 g or 100 g glucose tolerance tests. Two elevated glucose values of the 3-h glucose tolerance test or one elevated glucose value of 75 g OGTT is diagnostic of GDM. While the cutoff values of 2 h 75 g OGTT are determined as 92 mg/dl (5.1 mmol/L) for fasting, 180 mg/dl (10 mmol/L) for the first hour and 153 mg/dl (8.5 mmol/L) for the second hour; the threshold values of 100 g OGTT are determined as 95 mg/dl (5.3 mmol/L) for fasting, 180 mg/dl (10 mmol/L) for the first hour, 155 mg/dl (8.6 mmol/L) for the second hour, and 140 mg/dl (7.8 mmol/L) for the third hour (Table 1).

The Hyperglycemia and Adverse Pregnancy Outcome Study (HAPO) was a large, prospective-designed, observational study that included blinded pregnancy outcome data in women whose fasting plasma glucose level did not exceed 105 mg/dL (5.8 mmol/L) or the 2-h 75 g plasma glucose level did not exceed 200 mg/dL (11.1 mmol/L) or both (HAPO Collaborative Research Group 2008). Otherwise the values were unblinded to women and the caregivers. Primary outcomes were identified birth weight above 90th percentile, caesarean section delivery, neonatal hypoglycemia and cord C-peptide above 90th percentile (HAPO Collaborative Research Group 2008). According to the study findings a strong correlation was reported between increased maternal blood glucose levels and a range of adverse maternal and fetal outcomes (HAPO Collaborative Research Group 2008).

Abnormal values of blood glucose are associated with hypertensive disorders of pregnancy, increased rates of cesarean section, perineal trauma, placental abruption, postpartum infection and hemorrhage, and development of type 2 diabetes. On the other hand, perinatal mortality (intrauterine demise or neonatal death), large-for-gestational age (LGA) infants, neurosensorial disability, preterm birth, macrosomia, and lower scores of APGAR at 5 min are the leading short-term complications for fetuses whose mothers have complications with GDM (Raman et al. 2017). In utero excessive fetal glucose exposure affects cardiovascular and metabolic systems and results in increased long-term risk of obesity, diabetes mellitus and cardiac diseases during adult life (Carreiro et al. 2018) (Table 2).

Once a pregnant woman is diagnosed with GDM, an appropriate initiation of active treatment including diet, exercise, and medical treatment (oral anti-diabetics and/or insulin) has to be arranged. Besides, blood glucose monitoring is very important to understand the success and follow-up of the treatment and requirement

Table 1 Cutoff values for diagnosing gestational diabetes mellitus

	75 g OGTT[a]		100 g OGTT[a]	
Fasting glucose	92 mg/dl	5.1 mmol/L	95 mg/dl	5.3 mmol/L
1-h glucose	180 mg/dl	10 mmol/L	180 mg/dl	10 mmol/L
2-h glucose	153 mg/dl	8.5 mmol/L	155 mg/dl	8.6 mmol/L
3-h glucose	NA		140 mg/dl	7.8 mmol/L

[a]For 75 g OGTT, at least one value meeting the threshold is diagnostic for GDM. For 100 g OGTT, at least two values meeting the threshold are the diagnostic for GDM

Table 2 Maternal, fetal, and neonatal complications of gestational diabetes mellitus

Maternal complications	Fetal and neonatal complications
• Preeclampsia	• Prematurity
• Cesarean section	• Perinatal asphyxia
• Operative deliveries	• Respiratory distress
• Postpartum hemorrhage	• Perinatal mortality (intrauterine demise or neonatal death),
• Perineal trauma	• Large-for-gestational age infants
• Placental abruption	• Neurosensorial disability
• Postpartum infection	• Macrosomia
• Development of type 2 diabetes	• Lower scores of APGAR at 5 min

of additional intervention to avoid maternal and fetal adverse outcomes. Blood glucose monitoring suggestions regarding treatment targets are for self-measured capillary blood glucose levels. The accuracy of these measurements depends on multiple factors. The glucose measurements after 1 week of monitoring and every 2–3 weeks thereafter until delivery should be checked and the patients should be informed if any changes to treatment are required (Kaiser Foundation Health Plan of Washington 2021).

Noncontinuous Versus Continuous Glucose Monitoring:

There are two types of glucose monitoring techniques with self-assessment: non-continuous and continuous glucose monitoring (Gonzales et al. 2019). Non-continuous glucose monitoring (NCGM) is called as self-monitoring and this technique aims to monitor the blood glucose levels in a frequency during a day depending on diabetes type, and the type of medical treatment. However, continuous blood glucose monitoring (CGM) assesses the glucose levels automatically every few minutes. Even if CGM assessments need calibration at least twice daily, it has an advantage about the monitorization of rapid changes of blood glucose during a day. This technique is especially recommended by the American Diabetes Association (ADA) for type 1 diabetes patients with poor control (Professional Practice Committee 2018). However, the latest articles have shown that the continuous blood glucose monitorization can be used for blood glucose assessment in pregnancy complicated by GDM (Lane et al. 2019; Singh et al. 2020).

Self-monitorization of blood glucose can be done in two methods, and technically they are two separate modalities. NCGM devices are glucometers requiring finger puncture to access the capillary blood. The test strips generally contain an enzyme that the glucose oxidizes and three electrodes that are reference, counter, and working electrodes (Gonzales et al. 2019). Continuous glucose monitoring system works in a different way from NCGM. There are three crucial parts of the CGM devices: a transmitter, a sensor, and a wireless receiver (Gonzales et al. 2019). The sensor that is located under the skin is attached to the transmitter that transmits the measurements to the receiver via radiofrequency waves. At the end of the process, the receiver calculates the blood glucose level.

In literature, the studies that have focused on whether CGM has any effect on improving maternal and fetal adverse outcomes or not, have reported that while CGM was easy to use for the patients and beneficial for self-glycemic control; there were no differences in any maternal fetal outcomes between CGM use and NCGM (Yu et al. 2019; Kestilä et al. 2007; Alfadhli et al. 2016; Wei et al. 2016). Yu et al. reported better fetal outcomes in patients who use CGM in terms of birth weight, neonatal hypoglycemia and hyperbilirubinemia, and respiratory distress syndrome (2014). On the other hand, lower preeclampsia incidence has been reported in the CGM group by Voormolen et al. (2018). In a recent meta-analysis García-Moreno showed that women with GDM using CGM may achieve lower average blood glucose levels, lower maternal weight gain, and infant birth weight than women using NCGM (2021).

Postprandial Versus Preprandial Glucose Monitoring:

Noncontinuous glucose monitoring is the well-known and traditional way that aims at the optimal glycemic control. It has been shown that optimal glycemic control is important to reduce the incidence of adverse maternal and fetal outcomes. Especially, elevated postprandial glucose levels are associated with LGA babies than fasting glucose levels. However, optimal timing for postprandial glucose monitorization is still controversial. According to the latest guidelines about timing of glucose levels either 60th or 120th minutes after meals is favorable in pregnant women complicated with GDM (Zhang et al. 2019). Both ADA and ACOG have recommended that the cutoff levels of 140 mg/dl at the first hour and 120 mg/dl at the second hours are to be used as the blood glucose targets to reduce the risk of adverse fetal and neonatal outcomes (Jovanovic 1998).

The priority of optimal glycemic control in the GDM population is generally approved. But the optimal time for glucose assessment in GDM patients is still questionable. There are inconsistent results from the studies comparing fasting and postprandial measurements (de Veciana et al. 1995; Durnwald et al. 2011).

De Veciana et al. reported that postprandial follow-up is more advantageous in patients with GDM using insulin for reducing complication related to GDM (de Veciana et al. 1995). There are some studies reporting that neither pp1 (pp 60th minute) nor pp2 (pp 120th minute) glucose measurements has superiority to each other (Moses et al. 1999; Weisz et al. 2005). Some studies have reported no difference between postprandial 1-h and 2-h analyses (Moses et al. 1999; Weisz et al. 2005; Sivan et al. 2001). Furthermore, a different measurement (1 h after breakfast and 2 h after dinner) has been proposed as an option for the control of plasma glucose levels (Sivan et al. 2001).

Another issue of discussion is diurnal variation. Sivan et al. reported that plasma glucose level was higher at 60th minutes in the morning and 120th minutes in the evening (Sivan et al. 2001). The author proposed daily physical activities and meal composition to be responsible for the differences (Sivan et al. 2001). Besides, postprandial glucose peak was measured at the 90th minute by continue

monitorization in diabetic pregnancy (Ben-Haroush et al. 2004). Ben-Haroush et al. measured plasma glucose levels in diabetic pregnant women with poor glycemic control and showed that the increase to the highest levels in plasma glucose had occurred in the 90th minute (Ben-Haroush et al. 2004). Sever et al. proposed that postprandial 90th minute after breakfast, postprandial 60th minute after lunch, and postprandial 60th minute after evening have the highest plasma glucose values. They measured the highest plasma glucose value at the 90th minute after breakfast and at 60th minute after lunch and dinner (Fig. 1). They proposed a 90th-minute threshold value as 130 mg/dl (Sever et al. 2021). The latest recommendation about timing is either postprandial 60th minute or 120th minute glucose level is favorable to monitor plasma glucose levels in GDM pregnant women (ACOG 2018; American Diabetes Association 2014).

The ideal blood glucose monitoring protocol has to allow to detect the requirement of medical therapy for hyperglycemia and identification of hypoglycemia and hyperglycemia attacks. While deciding the most convenient technique and the frequency of blood glucose monitoring in a day, the clinician should look out for patients' comfort and compliance to the chosen protocol. In literature, Langer et al. compared the effect of the frequency of glucose monitoring on adverse maternal and fetal outcomes (Langer et al. 1994). They categorized glucose monitoring as conventional (4 times daily preprandial and postprandial 120th minutes) and intensified management (7 times daily preprandial and postprandial 120th minutes for every

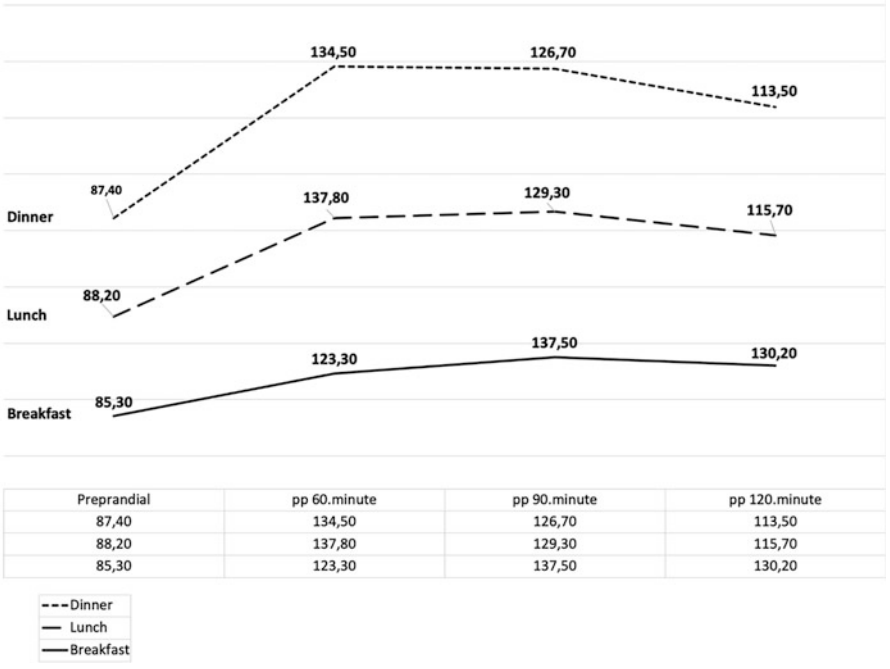

Fig. 1 Mean plasma glucose levels according to meals (Sever et al. 2021)

meal and bedtime). The authors reported that intensified glucose monitoring was more successful in recognizing inadequate treatment and decrease in maternal and neonatal complications (Langer et al. 1994). Sever et al. emphasized that they could not find a relation between perinatal complications and high plasma glucose levels except NICU requirements for postprandial 90th minute glucose values (2021). Goldberg et al. reported that daily glucose monitoring had better effect on controlling maternal glycemic status and identifying the patients who needed insulin treatment. The rates of LGA and macrosomia have been reported lower in daily glucose monitoring group than weekly glucose monitoring group (Goldberg et al. 1986). Moreover, the studies that focused on whether compliance to self-glucose monitoring advices associated with perinatal outcomes or not indicated that increased compliance to advices has been associated with decreased rates of adverse neonatal outcomes such as LGA and hypoglycemia (Wernimont et al. 2019).

According to Cochrane Database Systematic Reviews, when the authors compared the women who use preprandial and postprandial monitoring, there were no difference in terms of preeclampsia, caesarean section, perineal trauma, and hypoglycemia for newborn babies (Raman et al. 2017). However, the babies whose mothers used postprandial glucose monitoring were less likely to be born LGA than the babies whose mothers used preprandial monitoring.

Updated Guidelines

According to National Institute for Health and Care Excellence (NICE) guideline, the clinician should educate women with gestational diabetes how to self-monitor their blood glucose. Women with gestational diabetes who have multiple daily insulin injections have to test their fasting, premeal, postmeal, and bedtime blood glucose levels. If the women with gestational diabetes are being managed with diet and exercise, or oral antidiabetic agents, they should measure their fasting and 1-h post meal blood glucose levels (NICE 2015).

According to ACOG guideline, there is still insufficient evidence to define the optimal frequency of blood glucose monitoring (ACOG 2018). However, blood glucose measurement at four times a day, once at the time of fasting, and three times after each meal is recommended. Moreover, mean fasting glucose values are more valuable in managing gestational diabetes because fasting glucose levels are found more predictable for increased neonatal fat mass and macrosomia (ACOG 2018).

Canadian Diabetes Association guideline recommends that women with gestational diabetes should keep their fasting glucose <5.3 mmol/L (96 mg/dl), 1-h postprandial <7.8 mmol/L (140 mg/dl) and 2-h postprandial blood glucose <6.7 mmol/L (120 mg/dl) to avoid adverse pregnancy outcomes (Feig et al. 2018). Moreover, self-monitoring of fasting and postprandial blood glucose method should be performed to improve pregnancy outcomes (Feig et al. 2018).

American Diabetes Association (ADA) advises fasting and postprandial self-monitoring of blood glucose in women with gestational diabetes (American Diabetes Association 2020). Target glucose levels are determined for fasting <95 mg/dl, 1-h

postprandial glucose <140 mg/dl and 2-h postprandial glucose <120 mg/dl. On the other hand, the committee recommends not to use continuous glucose monitoring metrics to achieve optimal pre- and postprandial glycemic targets (American Diabetes Association 2020).

Society of Canadian Obstetrics and Gynecology recommends that women with pregestational or gestational diabetes mellitus should be provided with care by a multidisciplinary team intended to attain and preserve euglycemia (Berger et al. 2019). However, there is no more data about the frequency and target values of blood glucose levels during follow-up.

Self-monitoring of blood glucose is recommended by Scottish Intercollegiate Guidelines Network (SIGN) and International Diabetes Federation (IDF) for all pregnant women with gestational diabetes 3–4 times a day (fasting following at least 8 h after the last meal; postprandial 2–3 times daily – 1 or 2 h after the onset of the meal). Target blood glucose values are determined for fasting <5.3 mmol/L (96 mg/dl), 1-h < 7.8 mmol/L (140 mg/dl) and 2-h postprandial <6.7 mmol/L (120 mg/dl) (Scottish Intercollegiate Guidelines Network 2013; IDF Clinical Guidelines Task Force 2009).

Conclusion

This chapter describes the techniques of blood glucose monitoring, adverse maternal and fetal outcomes, and the content of updated evidence-based guidelines for gestational diabetes mellitus. Optimal glycemic control should be achieved to improve pregnancy outcomes and management of women with gestational diabetes mellitus. The clinician should keep in mind that the optimal glycemic control is crucial during prenatal care, intrapartum care, and postpartum care.

Mini Dictionary of Terms

- **Large-for-gestational age (LGA):** LGA refers to an infant or a fetus whose birth weight is above the 90th percentile or larger than expected for their age.
- **Preterm birth:** Preterm birth is defined as any birth before 37 weeks of gestation.
- **Macrosomia:** Fetal macrosomia is defined as the growth of the fetus beyond usually 4000–4500 g regardless of the gestational week.
- **APGAR:** An index to evaluate of newborn well-being. Appearance, pulse, grimace, activity, and respiration are used to calculate APGAR score.
- **Insulin:** A peptide hormone produced by beta cells of the pancreatic islets; it is considered to be the main anabolic hormone of the body.

Key Facts

- Gestational diabetes mellitus is defined as new-onset hyperglycemia and carbohydrate intolerance during pregnancy without a history of diabetes.

- Abnormal values of blood glucose are associated with hypertensive disorders of pregnancy, increased rates of cesarean section, perineal trauma, placental abruption, postpartum infection and hemorrhage, development of type 2 diabetes.
- Perinatal mortality (intrauterine demise or neonatal death), large-for-gestational age (LGA) infants, neurosensorial disability, preterm birth, macrosomia, and lower scores of APGAR at 5 min are the leading short-term complications for fetuses whose mothers are complicated with GDM.
- Once a pregnant woman is diagnosed with GDM, an appropriate initiation of active treatment including diet, exercise, and medical treatment (oral antidiabetics and/or insulin) has to be arranged.

Summary Points

- The diagnosis of GDM is based on the results of a 75 g or 100 g glucose tolerance tests. One elevated glucose value of 75 g GTT or two elevated values of 100 g GTT is diagnostic.
- Blood glucose monitoring is the essential part of the management of a patient who is complicated by GDM. It can be assessed as continuous/non-continuous and pre-/postprandial blood glucose levels.
- American Diabetes Association (ADA) advises fasting and postprandial self-monitoring of blood glucose in women with gestational diabetes. Target glucose levels are determined for fasting $<$95 mg/dl, 1-h postprandial glucose $<$140 mg/dl and 2-h postprandial glucose $<$120 mg/dl.
- The optimal glycemic control should be achieved to improve pregnancy outcomes and management of women with gestational diabetes mellitus.
- The clinician should keep in mind that the optimal glycemic control is crucial during prenatal care, intrapartum care and postpartum care.

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Metabolomic Biomarkers, Metabolite Patterns, and Gestational Diabetes Mellitus

50

Ellen C. Francis and Wei Perng

Contents

Introduction 1034
Epidemiology, Etiology, and Consequences of Gestational Diabetes Mellitus 1035
Epidemiology 1035
Etiology 1035
Consequences 1036
Screening and Diagnosis 1037
Metabolic Heterogeneity Among GDM Cases 1038
Applications of Metabolomics in Gestational Diabetes Mellitus 1039
Prevention/Prediction 1039
Treatment/Prognosis 1041
Future Directions 1045
Mini-Dictionary of Terms 1046
Key Facts of Gestational Diabetes Mellitus 1047
Summary Points 1047
References 1047

Abstract

Gestational diabetes mellitus (GDM) is a common pregnancy complication associated with risk of type 2 diabetes (T2D) for mother and offspring. Although glucose intolerance is a hallmark of GDM, there is evidence of GDM subtypes. Here we outline the application of metabolomics to understanding risk, treatment, and prognosis of GDM, and where possible the utility of metabolomics in understanding subtypes of GDM. Studies of metabolomics using blood collected during pregnancy indicate that GDM risk and treatment is associated with altered levels of amino acids, long-chain fatty acids, and glycolytic intermediates.

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Further, use of multiple metabolites in conjunction with clinical factors has a better value over clinical factors alone or a single metabolite, for predicting risk of T2D among women who had GDM. Although these data provide novel insights, the application of metabolomics in guiding GDM treatment, monitoring of adherence, and evaluation of prognosis has yet to be fully realized.

Keywords

Gestational diabetes mellitus · Metabolomics · Hyperglycemia · Insulin sensitivity · Insulin secretion · Pregnancy · Type 2 diabetes · Amino acids · Lipids

Abbreviations

1,5-AG	1,5-anhydroglucitol
AUC	Area under the curve
BCAA	Branched-chain amino acids
BMI	Body mass index
GCT	Glucose challenge tests
GDM	Gestational diabetes mellitus
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment for insulin resistance
LCFA	Long-chain fatty acids
LPC	Lysolecithin
LPE	Lysocephalin
MS	Mass spectrometry
NEFA	Nonesterified fatty acids
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
PC	Phosphatidylcholines
PE	Phosphatidylethanolamine
T2D	Type 2 diabetes

Introduction

During pregnancy, maternal resource allocation is governed by complex physiological adaptations. An array of pregnancy complications are associated with suboptimal adaptive responses to pregnancy and may result in higher risk of detrimental short- and/or long-term health outcomes for both the mother and offspring (Hernandez et al. 2020; McIntyre et al. 2019). Of particular concern in developed countries and the focus of this chapter is gestational diabetes mellitus (GDM), a pregnancy complication with heterogeneous etiology that affects anywhere from 5% to 13% of women globally (Zhu and Zhang 2016), and continues to increase in prevalence as a consequence of the obesity epidemic.

In this chapter, we describe the epidemiology and heterogenous pathophysiology of GDM, followed by a targeted review of the literature and discussion of the

applications of metabolomics profiling to better understand risk, biomarkers, and prognosis of this pregnancy complication. Although metabolomics profiling can be implemented in any tissue type, we focus on metabolomics assayed from maternal blood given the relatively noninvasive nature of obtaining this biospecimen and the fact that blood is a systemic tissue that reflects, to some extent, all ongoing metabolic processes.

Epidemiology, Etiology, and Consequences of Gestational Diabetes Mellitus

Epidemiology

GDM is characterized by glucose intolerance with first recognition or onset occurring during pregnancy (American Diabetes Association 2010). The prevalence of GDM varies by geographic region mainly based on the distribution of common risk factors such as age, parity, race/ethnicity, and obesity, as well as differences in diagnostic criteria (Zhu and Zhang 2016). GDM is the most common metabolic condition of pregnancy, and likely reflects the increasing prevalence of obesity, diabetes, higher maternal age among primigravida women, and sedentary lifestyles (Bardenheier et al. 2015). Moreover, the relationship between risk factors and GDM is cyclical as women who are diagnosed with GDM are also at increased risk for subsequent obesity and diabetes. Therefore, the increase in GDM is both a consequence of and contributor to the increasing prevalence in these chronic conditions (Ferrara 2007).

Etiology

The pathophysiology of GDM is centered around inadequate insulin secretion in response to increased maternal insulin resistance, leading to hyperglycemia that is detected by routine obstetric screening in pregnant women. During pregnancy, there are numerous metabolic changes that govern the allocation of maternal-fetal resources. These changes are marked by progressively greater insulin resistance in the second and third trimesters, which are thought to be secondary to increases in maternal adiposity, and placental and other hormones (Barbour et al. 2007). In instances where the beta-cells are unable to compensate for the increased insulin requirements in the latter half of pregnancy, women develop hyperglycemia and, at the higher end of the spectrum, GDM.

Despite the shared hallmark of hyperglycemia during pregnancy, the pathophysiology of GDM is heterogeneous. GDM transpires from a complex set of interactions between genetic and environmental factors, triggered by metabolic challenges of pregnancy (e.g., adipose tissue accretion in early gestation and lipolysis in late pregnancy [McIntyre et al. 2019]). A major risk factor for GDM is overweight or obesity prior to pregnancy, as women with obesity have an elevated insulin response

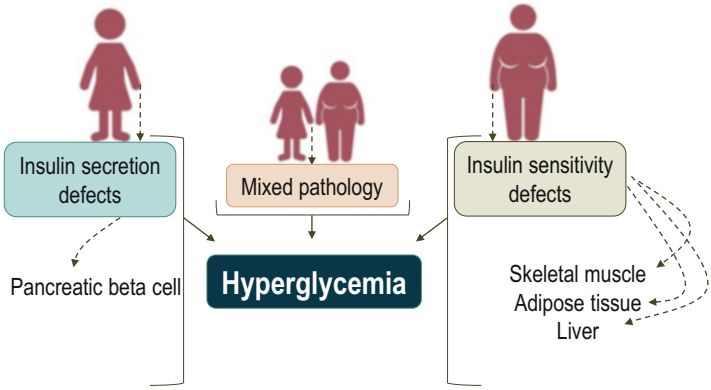

Fig. 1 General overview of etiological subtypes of hyperglycemia in pregnancy. This figure shows a general overview of different GDM subtypes. However, GDM may be caused by a complex set of interactions between genetic and environmental factors, triggered by a series of metabolic challenges during pregnancy (e.g., adipose tissue accretion in early gestation and lipolysis in late pregnancy)

in early pregnancy (Zhang and Ning 2011). It is hypothesized that the insulin response during early pregnancy among women with obesity leads to early placental growth, and release of placental factors that further decrease insulin sensitivity in maternal tissues (Catalano and Shankar 2017). As such, the prevalence of GDM varies within strata of weight status, with 2–5% of normal weight women and 6–12% of women with overweight/obesity diagnosed with GDM (Kim et al. 2010).

The difference in adiposity status among women with GDM likely reflect different etiologies, such that women with obesity and GDM have hyperglycemia due to deficiencies in insulin sensitivity, whereas women without obesity have hyperglycemia predominantly characterized by deficiencies in insulin secretion (Fig. 1) that likely preceded conception but went undetected until the greater insulin demands of pregnancy emerged.

Consequences

There are numerous short- and long-term health consequences of GDM that effect both women and their offspring. In the short term, GDM is associated with overnutrition and excessive fetal growth, which in turn can increase the risk of shoulder dystocia, birth trauma, operative delivery, preterm delivery, and neonatal hypoglycemia (International Association of Diabetes Pregnancy Study Groups Consensus Panel et al. 2010; Metzger et al. 2007). The long-term maternal health outcomes include risk for developing obesity, diabetes, cardiovascular disease, and hypertension later in life (O'Sullivan and Mahan 1964; Daly et al. 2018; Retnakaran 2018). Additionally, human and animal studies indicate that exposure to elevated levels of glucose in utero induces modifications to fetal insulin metabolism, ultimately

increasing the offspring's risk of metabolic disorders later in life (Aerts and Van Assche 2006; Plagemann 2005). Epidemiological studies from human cohorts have reported that exposure to prediabetes, diabetes, or GDM is associated with marked increases in risk of child obesity, T2D, and metabolic syndrome in adult offspring (Clausen et al. 2008, 2009, 2013; Dabelea et al. 2000; Francis et al. 2020; Lowe et al. 2018). These findings support the adverse impact of in utero exposure to hyperglycemia on offspring adiposity and glucose-insulin homeostasis.

Screening and Diagnosis

Assessment of risk factors for quantifying the true impact of GDM is complicated by a lack of consensus on diagnostic criteria for this condition (discussed in Long and Cundy (2013); McIntyre et al. (2014)). For instance, a two-step approach is still common practice in the USA, whereas other organizations both in the USA and internationally have adopted a one-step approach, which is considered less conservative (Table 1). For a detailed discussion of global GDM prevalence and differences in diagnostic criteria, see Zhu and Zhang (2016).

Many women are not screened for diabetes prior to becoming pregnant, inevitably resulting in a proportion of gravidas with unrecognized hyperglycemia prior to pregnancy. Thus, most organizations differentiate between women who have GDM (a less severe level of hyperglycemia) versus those who have "overt" diabetes during pregnancy (fasting plasma glucose >126 mg/dl or random plasma glucose

Table 1 Screening and diagnosis of GDM based on plasma or serum glucose values mg/dL

Two step	50 gram GLT screen	Fasting	OGTT	1 hour	2 hour	3 hour
NIH consensus* Conversion CC/NDDG	140	95/105	100 g	180/190	155/165	140/145
One step						
IADPSG**	NR	92–125	75 g	180	153–199	NR

For the two-step approach all women are screened at 24–28 gestational weeks with a nonfasting 50 g glucose load test (GLT). If after 1 hour plasma glucose concentrations are ≥140 mg/dL, women proceed to a fasting 100 g 3 hour oral glucose tolerance test (OGTT) (step two). In general, the diagnosis of GDM requires at least two elevated OGTT values (ACOG Committee on Obstetric Practice 2018). In contrast, the one-step approach does not screen women, and instead utilizes a fasting 75 g 2 hour OGTT for all women, and a diagnosis of GDM is made if at least one elevated OGTT value is present (International Association of Diabetes Pregnancy Study Groups Consensus Panel et al. 2010).

Abbreviations: International Association of Diabetes and Pregnancy Study Groups (IADPSG); National Institute of Health (NIH); Not Required (NR); Carpenter Coustan (CC); National Diabetes Data Group (NDDG); Glucose Load Test (GLT)

*Requires two elevated values, endorsed by the American Congress of Obstetricians and Gynecologists (ACOG) with any of the following thresholds for the 50 g GLT screen 130, 135, or 140 mg/dL; ** Requires one elevated value, endorsed by the American Diabetes Association (ADA); World Health Organization (WHO)

>200 mg/dl) (International Association of Diabetes Pregnancy Study Groups Consensus Panel et al. 2010; World Health Organization 2013). Screening for and diagnosis of GDM based on the maternal response to a glucose challenge test (GCT) or oral glucose tolerance test (OGTT) are used to stratify pregnant women and their fetus to differing degrees of obstetric risk. While fasting glucose, GCT, and OGTT provide insight into the degree of hyperglycemia, they do not inform on whether hyperglycemia is driven by deficits in insulin sensitivity, insulin secretion, or other insulin signaling disruptors such as inflammation (Kirwan et al. 2002; Powe et al. 2020). Details on the drivers of hyperglycemia during pregnancy are an important area for future research and treatment efforts.

Metabolic Heterogeneity Among GDM Cases

Metabolic heterogeneity associated with the physiologic subtypes of GDM described above has been related to overlapping and distinct offspring health outcomes (Layton et al. 2019; Powe et al. 2016; Powe et al. 2021; White et al. 2020). GDM characterized by insulin resistance – which overlaps substantially with maternal overweight/obesity – has been associated with greater risk of large-for-gestational age, high birthweight, and neonatal hypoglycemia even after accounting for differences in maternal body mass index (BMI) (Powe et al. 2016). Emerging evidence indicates a detrimental effect of maternal hyperglycemia on offspring metabolism, even below thresholds of GDM (Francis et al. 2021; Heslehurst et al. 2007; Metzger et al. 2008; Perng et al. 2017; Perng et al. 2020; Shokry et al. 2019). Accordingly, such adverse offspring outcomes are apparent even among women with an abnormal GCT but who did not develop GDM based on a later 2 h OGTT (Selen et al. 2021). On the other hand, for cases of GDM characterized by beta-cell insufficiency – a suspected cause of GDM among women who are not overweight/obese prior to pregnancy – perinatal outcomes show a similar trend to those among women with normal glucose tolerance (Liu et al. 2018; Powe et al. 2016; Selen et al. 2021). However, examining short- and long-term outcomes among subtypes of GDM has only just begun to be undertaken and findings from these few studies should not be taken as conclusive.

To understand the long-term maternal health risks within subtypes of GDM (i.e., driven by excess adiposity and insulin sensitivity deficiency vs. driven by beta-cell defects), polygenic risk scores for type 2 diabetes (T2D) have been used (Powe et al. 2019). Interestingly, although GDM with predominant insulin sensitivity deficits was associated with adverse short-term outcomes as discussed above, GDM driven by beta-cell insufficiency/insulin secretion deficiency, or a mix of the two, was associated with an increased genetic risk of T2D (Powe et al. 2019). In general, genetic variants associated with beta-cell function and lipid metabolism represent shared underpinnings between GDM and T2D (Ding et al. 2018; Kwak et al. 2012; Powe et al. 2021). However, effect sizes of genetic variants in relation to the risk of GDM tend to be relatively small (Berkowitz 2020; Ding et al. 2018; Kwak et al. 2012; Powe et al. 2021), and are not always related to the maternal and child health

consequences of GDM (Powe et al. 2021). Thus, there has been increased interest in new technology that can better capture the physiological precursors and outcomes associated with GDM.

Applications of Metabolomics in Gestational Diabetes Mellitus

Over the past 20 years, there has been substantial interest in the utility of metabolomics – the study of small endogenous molecules (metabolites) in biological tissue – for investigating the causes and consequences of GDM. This interest stems from the ability of metabolomics to capture the molecular phenotype that reflects both internal physiological conditions shaped by upstream "omics" (genomics, epigenomics, transcriptomics, and proteomics), as well as external exposures ranging from environmental toxicants to lifestyle behaviors. Metabolomics studies are broadly categorized as untargeted, which result in data on the relative concentrations of all measurable analytes within a biological sample, or targeted, which quantifies the physiological levels of a specific list of compounds of a priori interest and includes internal standards to derive absolute concentrations of each metabolite (McCabe and Perng 2017). In the following sections we discuss applications of metabolomics in GDM prevention/prediction, and treatment/prognosis.

Prevention/Prediction

Early identification of women at risk of developing GDM can help providers counsel women on adoption of healthy behaviors to circumvent progression to more severe hyperglycemia. However, as outlined above, GDM is a complex and heterogeneous pregnancy complication, and the use of metabolomics to distinguish between the etiological underpinnings of GDM is in nascent stages (Layton et al. 2019).

Among studies with maternal blood (serum/plasma) collected prior to GDM diagnosis (Bentley-Lewis et al. 2015; Chen et al. 2010; de Seymour et al. 2014; Diaz et al. 2011; Law et al. 2017b; Pinto et al. 2015), the data indicate that altered circulating levels of certain amino acids (e.g., alanine, glutamine/glutamate, betaine, and creatine metabolism) (Bentley-Lewis et al. 2015; Diaz et al. 2011; Pinto et al. 2015), long-chain fatty acids (LCFA) (e.g., palmitoleic and myristic) (Chen et al. 2010), and glycolytic intermediates (e.g., pyruvate and lactate) (Pinto et al. 2015) (Fig. 2) may be hallmarks of GDM risk. In addition, lower levels of phospholipids rich with unsaturated fatty acids have also been noted among women who develop GDM (Law et al. 2017b), whereas higher concentrations of glycolipids have been associated with higher GDM risk (Lu et al. 2016; Rahman et al. 2021b). Although higher levels of branched-chain amino acids (BCAA)s have been implicated in disruptions to glucose-insulin homeostasis and risk of T2D (Tobias et al. 2018), only one study conducted prior to GDM diagnosis found higher levels valine at 16–21 gestational weeks and risk of GDM (Pinto et al. 2015).

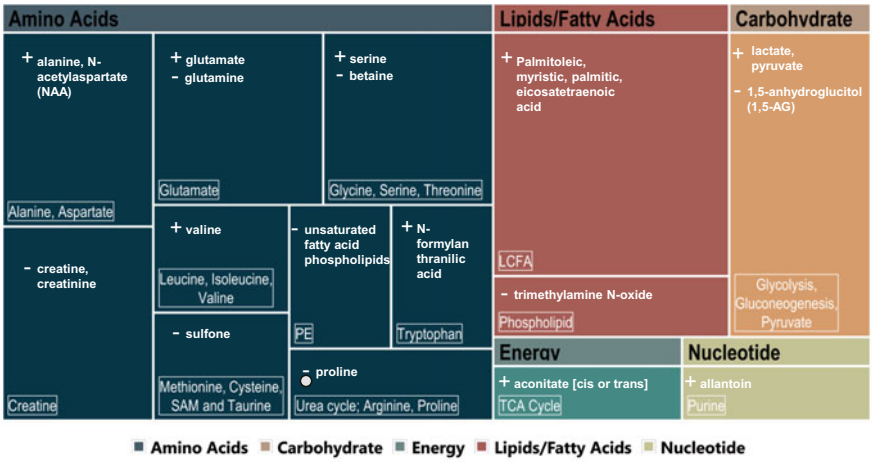

Fig. 2 Overview of metabolites in early pregnancy associated with increased GDM risk. Data are from studies where blood was collected prior to diagnostic screen for GDM. Data are presented as superclass, subclass, and biochemical name with size of box indicating great metabolites identified in that class. + positive association; − negative association

Despite differences in the platform approach (untargeted vs. targeted) and analytical instrumentation (nuclear magnetic resonance [NMR] vs. mass spectrometry [MS]), timing of blood collection (first vs. second trimester), study population (American, European, and Asian), and diagnostic criteria (IADPSG vs. CC), alterations in amino acid, LCFA, and glycolysis pathways appear to precede a diagnosis of GDM. These findings align with metabolomics analyses of obesity and insulin resistance from studies in nonpregnant populations (Gonzalez-Franquesa et al. 2016), and suggest that perturbances in amino acid and fatty acid metabolism contribute to development of deficiencies in insulin sensitivity during pregnancy. However, whether this contributes to insulin sensitivity irrespective of etiological differences in GDM is yet to be determined.

An additional utility of metabolomics is that it also captures sources of metabolic heterogeneity from external (nonphysiological) factors including but not limited to environmental exposures, social circumstance, and lifestyle behaviors, and thus has been used to discriminate women who will develop GDM regardless of demographic characteristics. However, the extent to which these metabolites add predictive value beyond conventional risk factors for GDM such as obesity, family history of diabetes, and race/ethnicity requires formal assessment. In a study conducted among 357 racially homogenous Finnish women with overweight/obesity, 18% of whom had a family risk of diabetes, small high-density lipoprotein (HDL) particles had the greater value for predicting GDM than prepregnancy BMI (area under the curve [AUC] [95% CI]: 0.71 [0.66, 0.77] vs. 0.56 [0.48, 0.63]) (Mokkala et al. 2020). Similarly, higher levels of small HDL particles in early pregnancy (15–18 gestational weeks) clearly distinguished women who developed GDM among a

racially diverse sample of 646 obese women from the UK (White et al. 2017). These data demonstrate the power of metabolomics to provide novel insights into the features of disease onset, beyond conventional assessments of risk. Future studies are warranted to replicate studies like these and determine whether the application of a single class of metabolites has clinical utility beyond usual clinical assessments.

Treatment/Prognosis

The application of metabolomics in guiding GDM treatment, monitoring of adherence, and evaluating prognosis has yet to be realized. We propose that an important first step is using metabolomics to profile women at or near the time of GDM diagnosis to obtain a better understanding of the metabolic profile of GDM which has implications for associated complications and long-term outcomes.

Amino Acid Profile

Recently, a spotlight has been turned on BCAAs (leucine, isoleucine, and valine) and their metabolic by-products in relation to GDM. This is due to growing evidence that BCAAs are associated with markers of glucose-insulin metabolism (including T2D), and improve prediction of incident T2D above and beyond traditional risk factors (e.g., BMI and fasting glucose) (Lynch and Adams 2014; Wang et al. 2011; Würtz et al. 2013). Most metabolomics studies conducted at the time of GDM diagnosis or later in pregnancy have identified differences in amino acid metabolism among women with GDM, and some but not all have identified higher levels of BCAAs among women with GDM (Dudzik et al. 2014; Hajduk et al. 2015; Lehmann et al. 2015; Liu et al. 2016; Scholtens et al. 2014). A recent meta-analysis demonstrated that women with GDM had significantly higher levels of all three BCAAs (standardized mean difference [SMD] [95% CI]: leucine = 3.76 [1.70, 5.82]; isoleucine = 3.15, [1.42, 4.87]; valine = 2.77 [1.21, 4.32]) compared to normoglycemic women (Zhao et al. 2019). Beyond BCAAs, higher levels of other amino acids including alanine, aspartate, creatine, and tyrosine, as well as alterations in glutathione metabolism have been observed in women with GDM compared to normoglycemic controls (Dudzik et al. 2014; Hajduk et al. 2015; Liu et al. 2016; Scholtens et al. 2014). Lower levels of compounds from the metabolism of glycine, serine, threonine, histidine, and tryptophan have been shown to contribute to the profile of women with GDM (Dudzik et al. 2014; Law et al. 2017a; Lehmann et al. 2015; Leitner et al. 2017; Liu et al. 2016). Taken together, current evidence indicates that GDM is associated with altered activity in pathways of amino acid metabolism and that future work is needed to understand if targeting BCAAs and other amino acids can improve glucose metabolism.

Amino Acids and Applications to GDM Treatment

Although insulin sensitizing medications (e.g., metformin) decrease alanine and phenylalanine in nonpregnant overweight/obese individuals with impaired fasting glucose (Irving et al. 2015), these findings do not completely align with emerging

data in pregnant women. A randomized controlled trial comparing insulin vs. metformin among women with GDM reported lower glutamine and higher phenylalanine at 22–24 gestational weeks (prior to treatment assignment) among women who went on to require medication compared to women who were able to maintain glucose levels with diet changes alone (Huhtala et al. 2018). Although glucose control was achieved in both treatment groups, BCAA isoleucine (median 11% vs. 5%, $P = 0.04$) and alanine (mean 16% vs. 8%, $P < 0.01$) had increased more by 36 gestational weeks in the metformin group. In this study, when insulin and metformin groups were combined, alanine levels at 36 gestational weeks were positively associated with birth weight and this effect was mostly explained by the 83% stronger association among those randomized to metformin. In another trial among overweight/obese pregnant women, an NMR metabolomics platform was used to analyze the serum metabolite profile of women treated with diet only versus those treated with insulin, metformin, or both (Mokkala et al. 2020). Although all treatment groups had significant reductions in glucose concentrations, there remained significant differences in the amino acid metabolomics profile of women treated with medication. Taken together, if BCAAs and other amino acids are implicated in the health outcomes associated with GDM, comprehensive treatments that target more than glucose levels are needed. However, it is important to note that in both studies no comparisons of the birth outcomes among the diet-controlled GDM group were made and thus whether lifestyle interventions impact BCAAs was not reported.

Differences in metabolite profiles following differing treatment regimes with the shared goal of reducing glucose levels may reflect treatment-specific effects on metabolism as well as heterogeneity in GDM etiology. With respect to the latter, women who require insulin sensitizing treatment such as metformin tend to represent a group that aligns more closely with deficits in insulin sensitivity as these women typically have greater adiposity (Kelley et al. 2015), and it is plausible that these differences remain apparent in circulating metabolites even after treatment. Although current clinical practice focuses on normalizing glucose levels, metabolomics and amino acid profiling of women treated with medication indicate that this glucose-centric treatment approach does little to impact the other components of their aberrant metabolic profile. This may have consequences for later health outcomes such as progression to T2D (Lynch and Adams 2014). Whether BCAA or metabolites from other classes of amino acids can be used as indicators of ideal candidates for treatment response during pregnancy requires further study.

Amino Acids and Applications to GDM Prognosis

Metabolomics has been applied with the objective of identifying early diagnostic biomarkers, predictors, and insight into the transition from GDM to T2D. In one of the first studies to use metabolomics in prediction of progressing from GDM to T2D over 2 years of follow-up, several BCAAs and 2-aminoadipic acid were significantly elevated at 6–9 weeks postpartum among women who later developed T2D (Allalou et al. 2016). When metabolites in the amino acid class were combined with other metabolite classes in a predictive model, the accuracy of prediction was superior to

that of a model that included clinical parameters including BMI, fasting glucose, insulin, and homeostatic model assessment for insulin resistance (HOMA-IR) (AUC: 0.77 vs. 0.71). In a follow-up study within this same cohort, changes in metabolites across the 2 years of follow-up were examined to assess the metabolic paths preceding T2D onset (Lai et al. 2020). Amino acid metabolites decreased in all women over follow-up; however, there was a smaller decrease in these metabolites among the incident T2D cases, suggesting that higher levels of these compounds are associated with greater risk of progression to T2D. Similarly, higher levels of BCAAs have been linked to glucose disorders (prediabetes and T2D) in a study using blood collected during pregnancy (Liu et al. 2021). In this study, independent of risk factors for T2D such as family history of diabetes, maternal age, smoking, alcohol use, and BMI, the ratio of leucine/isoleucine in pregnancy was positively associated with 2 hr glucose, HOMA-IR, and disorders of glucose metabolism (prediabetes and T2D) 10–14 years later. While preliminary, these data indicate that BCAAs levels in pregnancy and postpartum improve the prediction of incident T2D among women with a history of GDM, beyond the usual clinical parameters.

Lipid Profile

Prior studies point to differences in the maternal lipid profile in association with disrupted glucose-insulin homeostasis during pregnancy (Ryckman et al. 2015). In general, metabolomics studies align with these findings and provide a more nuanced understanding of the lipid metabolism pathways involved. GDM has been associated with higher levels of 3-hydroxybutyrate, carnitine and its esters (Liu et al. 2021; Sun et al. 2020), medium- and long-chain acyl- and acetyl carnitines (Lehmann et al. 2015; Liu et al. 2016; Liu et al. 2021), and glycerolipids such as triglyceride (48:1, 51:1) (Lu et al. 2016; Rahman et al. 2021b). Studies have also reported associations of numerous phospholipid subclass such as phosphatidylcholines (PC) and phosphatidylethanolamine (PE), as well as their lysolecithin (LPC) and lysocephalin (LPE) by-products with GDM (Dudzik et al. 2014; Law et al. 2017b; Liu et al. 2016; Rahman et al. 2021a). However, the direction of association tends to differ based on whether the phospholipid is rich in unsaturated FAs (lower levels) and the type of acyl carbon double bond, with those having ester and ether linkages showing lower levels among women with GDM (Rahman et al. 2021b).

Lipids and Applications to GDM Treatment

Lipid profiles may be key to distinguishing some subtypes of GDM. Women with GDM characterized by deficits in insulin sensitivity had significantly higher triglycerides (2.20 vs. 1.82, $P = 0.002$) and higher nonesterified fatty acids (NEFA;0.34 vs. 0.24, $P < 0.0001$), whereas women with GDM and a predominant insulin secretion deficit differed from normoglycemic women with respect to NEFA (0.32 vs. 0.24, $P = 0.003$) only (Layton et al. 2019) despite the fact that the proportion of women who received insulin treatment did not differ by GDM subtypes. These data suggest that even among women with differing etiologies of GDM, a proportion may require insulin therapy regardless of subtype, or that more tailored treatment approaches are needed. In addition, traditional lipid biomarkers such as

total triglycerides may not provide the detail needed to potentially inform treatment within subtypes. In a study with metabolomics data at multiple time points in pregnancy (pre- and post-diagnosis of GDM), a sophisticated data reduction approach was used and an interconnected lipid network comprised of cholesterol esters, PCs, and long polyunsaturated triglycerides that was negatively associated with GDM was identified (Rahman et al. 2021b). Importantly, a higher score for this network was associated with lower c-peptide levels, which is a marker of insulin secretion and could indicate a stronger relationship of this lipid network with an insulin secretion deficit subtype. When analyses were stratified by GDM treatment, the negative association of this lipid metabolite network with GDM was only significant among women who received a diet and lifestyle intervention compared to antihyperglycemic medication. These findings suggest that lipid metabolites rather than traditional lipid biomarkers may facilitate early determination of treatment regime within subtypes of GDM.

Lipids and Applications to GDM Prognosis

Lipid imbalances during pregnancy may be markers for future T2D risk (Erion et al. 2016). A few recent studies have applied metabolomics using blood collected during pregnancy or postpartum to assess the relationship of lipid metabolites in the progression to postpartum hyperglycemia and T2D. Among women with blood collection 6–9 weeks postpartum, higher concentrations of triglyceride lipid species and lower levels of cholesterol esters, ceramides, FFA, lactosylceramides, PE, LPC, LPE, and sphingomyelin lipid species were associated with T2D risk during 2 years of follow-up (Khan et al. 2019). In contrast, among women with blood collected 12 weeks postpartum cholesterol esters, ceramides, and PCs all showed positive associations with incident T2D during 10 years of follow-up (Lappas et al. 2015), suggesting some amount of transiency either in metabolite profiles during the postpartum period and/or differential underlying causes of T2D occurring within 2 years versus within 10 years of pregnancy. Finally, in a study that implemented metabolomics profiling of fasting blood collected during pregnancy, long-chain acylcarnitines, acetylcarnitine, and 3-hydroxybutyrate were positively associated with fasting glucose and insulin resistance between 10 and 14 years postpartum (Liu et al. 2021). Yet, despite significant associations of individual metabolites with T2D risk, no single metabolite nor combination of metabolites improved prediction of incident T2D over follow-up when compared to clinical factors such as pre-pregnancy BMI or family history of diabetes. Moreover, in this study (Liu et al. 2021) and others (Khan et al. 2019; Lappas et al. 2015), when multiple metabolites from blood collected during pregnancy were pooled, there was minimal improvement in the prediction of a glucose disorder 10–14 years later (AUC: 0.71 vs. 0.73). The lack of improvement in predictive capacity could be due to several factors, the primary one being heterogeneity in the etiology of GDM which likely has implications for subsequent T2D risk. Other explanations include statistically significant but biologically irrelevant effect sizes of individual metabolites, and inappropriate analytical methods to identify metabolites of interest (Khan et al. 2019; Lappas et al. 2015).

Carbohydrates

Unsurprisingly, GDM is associated with differences in fasting and postprandial glucose levels, and glycemic control following GDM diagnosis is the determinant for whether antidiabetic medications or lifestyle interventions are prescribed. Some metabolomics studies have identified differences in metabolites from fructose classes and glycolysis among women with GDM (Dudzik et al. 2014; Pinto et al. 2015; Scholtens et al. 2014). Of increasing interest is 1,5-anhydroglucitol (1,5-AG) given its clinical utility in nonpregnant diabetes patients. In hyperglycemia, 1,5-AG competes for renal reabsorption resulting in greater urinary excretion and lower serum levels of 1,5-AG. In line with this, a study using metabolomics from blood collected at the time of GDM diagnosis reported lower levels of 1,5-AG among women with high levels of fasting glucose (Scholtens et al. 2014). However, due to a decrease in the threshold for glucose in the kidney during pregnancy it is possible that a reduction in serum 1,5-AG does not necessarily reflect glycemic control in pregnancy (Hashimoto and Koga 2015). Whether metabolomics can add to our knowledge of disturbances in carbohydrate metabolism associated with GDM, beyond pathways of glucose disposal, has yet to be determined. In fact, as previously mentioned, glucose levels and risk factors for insulin resistance (e.g., obesity) are the major determinants for GDM treatment and prognosis of short- and long-term complications ("Gestational Diabetes Mellitus," 2003).

Future Directions

Metabolomics has improved our knowledge of the etiology and pathogenesis of GDM. However, there is room for growth in its application and clinical relevance. First, given that GDM is a heterogeneous disease and is assessed with inconsistent diagnostic criteria, more research is needed to untangle differences in risk, etiology, and prognosis for differing subtypes of GDM. Second, differences in approaches to metabolomics profiling (targeted vs. untargeted), platform (MS vs. NMR), timing, and fasting state at sample collection make it difficult to reconcile differences in findings across studies (Playdon et al. 2019). Third, replication and validation of findings in populations with different distributions of GDM risk factors are needed. Complicating replication is a lack of uniformity in the statistical techniques applied in metabolomics. Considering the increasing number of computational tools being developed to analyze and interpret metabolomics dataset, a consensus on metabolomics methodology is greatly needed (Perng and Aslibekyan 2020). This latter point has made scientific inference difficult and stymied many researchers' efforts in using metabolomics to unveil novel biomarkers of GDM risk and prognostic predictors.

Future studies should consider the influence of dietary intake on metabolite levels, particularly amino acids and fatty acids. As such, studies assessing whether dietary therapeutics can alter the metabolites of interest outlined herein are needed. Similarly, research on other modifiable determinates of metabolites, such as physical

activity, could inform whether specific lifestyle behaviors differentially impact metabolite pathways, and thus support the creation of more tailored interventions. In addition, large-scale epidemiological studies with metabolomics profiling in target tissues directly relevant to in utero programming will enhance causal inference, and could assist in untangling the overlap in subtypes of GDM. For example, in a study where sphingomyelins were observed to be lower among women with GDM, mice with inhibited sphingolipid biosynthesis demonstrated impacts on late-phase insulin sensitivity pointing to disturbances in hepatic glucose metabolism (Khan et al. 2019). Studies of this nature can inform whether the functionality of tissues directly or indirectly involved in insulin resistance are impacted by disturbances in the metabolite pathways identified with metabolomics studies.

Finally, a few studies discussed in this chapter have combined metabolomics with proteomics (Hajduk et al. 2015) and lipodomics (Law et al. 2017b; Rahman et al. 2021b). Given the complexity of physiological processes, use of such multi-omics approaches will provide a more holistic view of metabolic processes involved in the etiology and prognosis of GDM. Future studies using multi-omics/integrative-omics approaches are an important next step in the application of metabolomics to identify key molecular drivers of glucose disturbances in pregnancy, though a critical first step is to invest in and develop methods for systems biological approaches. The strength of metabolomics is that it reflects both internal physiology and additional sources of metabolic heterogeneity from environmental exposures, social circumstance, and lifestyle behaviors. These strengths of metabolomics complement classic studies aimed at understanding causes and consequences of GDM which in turn may provide insight into the prevention, diagnosis, treatment, and prognosis of GDM.

Mini-Dictionary of Terms

- Gestational diabetes mellitus – a common pregnancy complication characterized by glucose intolerance with first recognition or onset in pregnancy.
- Hyperglycemia – high blood glucose.
- Insulin sensitivity – loss of cellular sensitivity to insulin in key tissues for disposal of glucose such as skeletal muscle, liver, and adipose tissue.
- Insulin secretion – the release of insulin from pancreatic beta-cells, primarily in response to glucose levels.
- Metabolomics – the study of small endogenous molecules (metabolites) in biological tissue.
- Heterogenous etiology – a medical condition with several root causes.
- Amino acid profile – leucine, isoleucine, valine, alanine, aspartate, creatine, tyrosine, alterations in glutathione, glycine, serine, threonine, histidine, and tryptophan metabolism.
- Lipid profile – 3-hydroxybutyrate, carnitine and its esters, medium- and long-chain acyl- and acetyl carnitines, glycerolipids, phospholipid subclass such as phosphatidylcholines, and phosphatidylethanolamine, as well as their lysolecithin and lysocephalin by-products.

Key Facts of Gestational Diabetes Mellitus

- Gestational diabetes mellitus (GDM) affects between 5% and 15% of pregnant women globally.
- A key feature of GDM is elevated glucose levels during the latter half of pregnancy; however, GDM is a heterogenous condition with key phenotypic differences in the predominate drivers of elevated glucose – namely deficits in insulin sensitivity, which is often associated with obesity, and deficits in insulin secretion.
- GDM is associated with short- and long-term health consequences for both the mother and offspring such as elevated risk of obesity and T2D.
- Metabolomics studies have shown that GDM is accompanied by differences in amino acid and lipid metabolites, and some of these differences are associated with women's risk of progressing to T2D postpartum.

Summary Points

- Screening for and diagnosis of GDM based on current diagnostic criteria do not provide insight whether hyperglycemia is driven by deficits in insulin sensitivity, insulin secretion, or a combination of both.
- Prior to GDM diagnosis, studies indicate that altered circulating levels of certain amino acids (e.g., alanine, glutamine/glutamate, betaine, and creatine metabolism), long-chain fatty acids (LCFA) (e.g., palmitoleic and myristic), and glycolytic intermediates (e.g., pyruvate and lactate) may be hallmarks of future GDM risk.
- Current evidence collected at GDM diagnosis indicates that GDM is associated with altered activity in pathways of amino acid metabolism and future work is needed to understand if targeting BCAAs and other amino acids can improve glucose metabolism and prognosis.
- Use of multiple metabolites in conjenction with clinical factors versus a single metabolite has a better predictive value for assessing future risk of T2D among women with a prior GDM pregnancy.
- The strength of metabolomics is that it reflects both internal physiology and additional sources of metabolic heterogeneity from environmental exposures, social circumstance, and lifestyle behaviors, which compliments classic studies aimed at understanding causes and consequences of GDM.
- The application of metabolomics in guiding GDM treatment, monitoring of adherence, and evaluating prognosis has yet to be realized, and an important first step is using metabolomics to profile women at or near the time of GDM diagnosis to obtain a better understanding of the metabolic profile of GDM which has implications for associated complications and long-term outcomes.

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Neuroinflammatory Biomarkers in Diabetic Encephalopathy: Linking Cholinergic and Cognitive Dysfunction

51

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Contents

Introduction 1055
Diabetes and Brain 1056
Neuroinflammation and Diabetes 1057
Mechanistic Insights Involved in Diabetes-Induced Neuroinflammation 1059
Cognitive Impairment and Neuroinflammation in Diabetic Animal Models 1063
Conclusion 1065
Application to Prognosis, Other Diseases, or Conditions 1065
Key Facts 1066
Mini Dictionary 1067
Summary Points 1067
References 1068

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Abstract

The progression of diabetes has been linked with disruption of the microvascular system, which may lead to other degenerative conditions, including diabetic nephropathy, cardiomyopathy, and neuropathy. Cognitive dysfunction has been linked with diabetes-induced neurodegeneration. Changes in metabolic processes, including disruption of inflammatory pathways, cholinergic function, purinergic processes, and neurotransmitters triggered by chronic hyperglycemia, may affect brain function. Hence, the need to explore novel biomarkers to identify the risk of neurological deficit and impairment in the central nervous system in diabetic conditions. This chapter thoroughly reviewed the recently published articles on the biochemical mechanisms and biomarkers associated with diabetic encephalopathy and neuroinflammation. The roles of the biomarkers involved in diabetic encephalopathy will provide novel insights on possible therapeutic interventions that can mitigate cognitive problems and neurological dysfunction associated with chronic hyperglycemia as well as diabetes mellitus.

Keywords

Diabetes · Diabetic encephalopathy · Cognitive impairment · Neuroinflammation · Cholinergic dysfunction

Abbreviations

AGEs	Advanced glycation end products
AP-1	Activator protein-1
APP	Amyloid precursor protein
ARG1	Arginase 1
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
CD36	Cluster of differentiation 36
CD74	Cluster of differentiation 74
CD86	Cluster of differentiation 86
CNS	Central Nervous System
COX-2	Cyclooxygenase 2
DM	Diabetes Mellitus
GSK-3	Glucose synthase kinase-3
HO-1	Heme oxygenase-1
HSP70	Heat shock protein 70
IDDM	Insulin-dependent diabetes mellitus
IGF1	Insulin-like growth factor 1
IL-17A	Interleukin 17A
IL-1rn	Interleukin 1 receptor antagonist
IL-1β	Interleukin 1β
IL-22	Interleukin 22
IL-4	Interleukin 4

IL4-Rα	Interleukin 4 Receptor alpha
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-9	Interleukin 9
iNOS	Inducible nitric oxide synthase
JNK	C-jun N terminal kinase
MAPK	A mitogen-activated protein kinase
Mrc1	Mannose Receptor C-Type 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear Factor kappa B
NIDDM	Non-insulin-dependent diabetes mellitus
NO	Nitric oxide
PKC	Protein Kinase C
PS1	Presenilin 1
ROS	Reactive oxygen species
STAT pathway	Signal transducer and activator of transcription pathway
TGFβ	Transforming growth factor Beta
TNF-α	Tumor necrosis Factor Alpha
VEGF	Vascular endothelial growth factor

Introduction

Diabetes mellitus (DM) is one of the most common metabolic disorders and has become a major public health burden (Mohamed et al. 2018). Currently, about 463 million people across the globe are living with diabetes and it has been projected to increase to 700 million by the year of 2040 (Saeedi et al. 2019). There has been an increase in the prevalence of the disease, especially in the middle- and low-income countries over the years. A recent report shows a 2.5% increase in the prevalence of diabetes annually in average in all over the world (Kotwas et al. 2021). Diabetes is characterized by high glucose levels in the blood, which is caused by the impairment in insulin secretion or its action (Kerner and Brückel 2014). The pathophysiological mechanisms of diabetes have revealed two major types, which include type 1 or insulin-dependent diabetes mellitus (IDDM) and type 2 or non-insulin-dependent diabetes mellitus (NIDDM) with the latter accounting for the most prevalent cases with more than 90% of total diabetic cases in the world (Guthrie and Guthrie 2004). It is developed due to the body's ineffective use of insulin and hence may be diagnosed mostly after forty years of age. It also has similar symptoms like type 1 diabetes and has been diagnosed increasingly in young children in recent years due to eating unhealthy diet, overweight, and obesity. On the other hand, type 1 diabetes is also primarily common in children and it is characterized by the dependence on insulin and requires regular or routine administration of insulin due to the deficiency in insulin production from pancreatic beta-cells (Li et al. 2017).

The pathogenesis of DM is linked with several risk factors, including genetics and environmental factors, sedentary lifestyle, lack of physical exercise, alcoholic

beverages, hyperinsulinemia, dyslipidemia, and low β-cell function (Kolb and Martin 2017). Other risk factors include oxidative damage to pancreatic β-cells, obesity, hyperuricemia, and pathogenic organisms (Tangvarasittichai 2015). The progression of diabetes has also been linked to some complications, including diabetic nephropathy, retinopathy, neuropathy, and diabetic foot disease (Chawla et al. 2016). Other diabetic complications that have been identified include hypertension, atherosclerosis, cardiovascular disease, coronary artery disease, ketoacidosis, liver cancer and pancreatic cancer, and diabetic encephalopathy (Petrie et al. 2018).

The brain is one of the sites of diabetic organ damage, commonly referred to as diabetic encephalopathy and linked to neurocognitive disruptions, memory problems, cognitive impairment, and behavioral problems (Moheet et al. 2015). Moreover, a strong association between neurological disorders and type 2 diabetes has been established compared to type 1 diabetes. Type-2 DM increases the risk of dementia, and this has been associated with motor dysfunction, attention deficit disorder, and memory impairment (Zilliox et al. 2016). Evidence shows that patients with type 2 diabetes may develop Alzheimer's disease or any dementia-associated conditions (Barbagallo and Dominguez 2014). The link between diabetes and cerebrovascular damage can be associated with different pathological mechanisms, including compromised immune and antioxidant defense systems and neuroinflammation, which are triggered mainly by the insulin resistance and chronic hyperglycemia (Ma et al. 2020). Some studies have shown the roles of pro-inflammatory biomarkers, blood–brain barrier (BBB), and redox imbalance in the pathogenesis of diabetes-induced cognitive decline and neurodegeneration. However, the pathological mechanisms involving diabetes-induced neuroinflammation and cognitive decline have not been extensively investigated. This chapter presents information on the biochemical mechanisms between diabetic-driven neuroinflammation and cognitive dysfunction. The highlighted mechanisms involved in diabetes-induced neuroinflammation will provide more insights to the pathogenicity of this diabetic complication and will supply possible therapeutic approach that can be explored to mitigate the progression of the disease.

Diabetes and Brain

The brain is one of the most complex organ in the body. It is an energy-intensive organ and consumes a lot of oxygen due to different metabolic reactions and functions in different regions of the brain (Bélanger et al. 2011). The brain derives its energy from glucose and consumes about 25% of the body's glucose required for vital functions and survival of the neurons (Mergenthaler et al. 2013). The continuous supply and consumption of glucose in the brain is important for metabolic activities, neuronal function, neurotransmission, and signal transduction (Blázquez et al. 2014). The brain is also sensitive to an important hormone (insulin) required for controlling glucose levels in the blood (Röder et al. 2016). Moreover, apart from the

regulatory activity of energy stores by insulin, it also plays a major role in cognition in the central nervous system (CNS). Insulin travels to the brain from the pancreas, the main site of production, through the blood–brain barrier with the help of insulin transporters (Banks et al. 2012). Insulin is also produced in neuronal cells, especially in the hippocampal, amygdala, prefrontal cortex, and hypothalamus regions of the brain (Blázquez et al. 2014). Some of its primary functions in the brain involve activation of insulin receptor signaling pathways, which facilitates the production of proteins required for enzymatic antioxidant defense system in the neurons, glucose metabolism, and neuroprotective and anti-neuronal death mechanisms. Other functions involve the improvement of neuronal plasticity (Hamed 2017). Furthermore, it also contributes in improving mitochondrial membrane potential, adenosine triphosphate (ATP) production, enhances NAD(P)H redox potentials and hexokinase activity, which affects glucose metabolism and neuronal modulation in the brain. Hence, hyperglycemia and hyperinsulinemia triggered in diabetic condition disrupts the function of insulin in the brain, which may contribute to brain atrophy and cognitive impairment (De La Monte and Suzanne 2012). Insulin resistance and hyperglycemia is also linked with impairment of neuronal plasticity, cognitive deficit, and behavioral problems in type 2 diabetes (Zilliox et al. 2016).

Long-term complications in the peripheral and central nervous system are one of the consequences of uncontrolled and poorly managed diabetic conditions (Hamed 2017). Apart from the fact that diabetes has been linked with multiple organ damage, a strong association has been established with impaired CNS function, cognitive decline, and memory impairment (De La Monte and Suzanne 2012; Zilliox et al. 2016). Experimental investigations involving neuroimaging studies showed that structural changes occur in different regions of diabetic brain (Hamed 2017). Cortical atrophy, white matter lesions, and lacunar infarcts were observed in the brain of diabetic patients (Moran et al. 2013). Furthermore, disruptions of metabolites associated with cognitive function, behavior, and neurotransmitters occur in the CNS of diabetic patients (Hamed 2017). The neuropathological mechanisms of diabetes-induced cognitive dysfunction have been explained to be multifactorial. Hence this has made the treatment and therapeutic approach for diabetes-associated cognitive dysfunction more cumbersome.

Neuroinflammation and Diabetes

Neuroinflammation has been identified as a pathological manifestation of chronic hyperglycemia induced by the activation of microglia and astrocytes, impaired pro-inflammatory pathways, and redox imbalance (Harry and Kraft 2008; Ahmad et al. 2022). Moreover, microglial activation in the brain's hippocampal region is associated with cognitive impairment and memory problems. Neuroinflammation involves the inflammation of cells and tissues in the central nervous system, which triggers neurotoxicity, neuronal degeneration, and cell death (Disabato et al. 2016). Hence, neuroinflammatory biomarkers have been identified as a contributory factor to cerebrovascular pathology and cognitive decline associated with diabetic

complications. The inability to produce insulin or insulin resistance leads to hyperglycemia, which activates the generation of reactive oxygen species (Galicia-Garcia et al. 2020).

Moreover, these changes have been shown to trigger pro-inflammatory biomarkers in the brain, which induce neuroinflammatory pathways, hence compromising microvascular tissues, including the blood–brain barrier, thereby allowing the entry of toxic substances that can induce end-organ damage, tissue abnormalities, brain lesions, and subsequent cognitive decline (Van Dyken and Lacoste 2018). The normal function of the blood–brain barrier is to protect the CNS against external substances that can cause neurovascular damage and abnormalities while giving access to the adequate influx of nutrients and metabolites required by the nervous system. However, disruption of the blood–brain barrier has been identified in diabetes mellitus. It is associated with the heightened generation of reactive oxygen species and glycation end products and upregulation of inflammatory biomarkers due to impaired glucose metabolism, which leads to cognitive decline, memory impairment, and ultimately dementia (Van Dyken and Lacoste 2018). A study by Rom et al. (2019) revealed that DM-induced neuroinflammation led to disruption of the blood–brain barrier, which triggered memory dysfunction in type 1 and 2 diabetic animal models. The upregulation of genes associated with pro-inflammatory pathways was observed in the brain of diabetic rats. Neuropathological studies also showed disruptions of the blood–brain barrier due to microglial activation and loss of pericyte coverage in the brain of diabetic animals compared to the control (Haruwaka et al. 2019; Elabi et al. 2021). The results also showed that hyperglycemia-induced upregulation or neuroinflammatory genes was associated with cognitive decline and compromise of the blood–brain barrier in the brain of diabetic rats model (Rom et al. 2019).

Most diabetic complications associated with the central nervous systems are mediated via inflammation. The level at which inflammation is induced by hyperglycemia in the brain is dependent on the region of the brain. For instance, neuroinflammation was induced in the midbrain in type 2 diabetic rats after 28 days (Huber et al. 2006). Hyperglycemia triggers a significant increase in mitochondrial respiration in astrocytes, pericytes, and endothelial cells, leading to redox imbalance and overproduction of mitochondrial ROS. The high levels of ROS produced activate NF-κB, activator protein -1 (AP-1) and STAT pathways, leading to upregulation of pro-inflammatory cytokine levels (Van Dyken and Lacoste 2018; Wang et al. 2012). The ROS produced attacks astrocytes and reduces the folding of gap proteins. ROS may also reduce pericyte coverage, impair BBB function, and cause cell degeneration (Brownlee 2005).

Furthermore, insulin resistance, hyperinsulinemia, and hyperglycemia contribute to elevated levels of proinflammatory biomarkers and acute phase reactants such as the interleukins, tumor necrosis factor, chemokines, C-reactive proteins, and cortisol (Benatti et al. 2016). Moreover, inflammation may also be triggered in the neurons due to impairment in the hypothalamus and adrenal axis, decrease in insulin sensitivity, and compromise in the immune system. The pro-inflammatory biomarkers participate in the progression of neurodegeneration and cognitive problems (Navarro

and Mora 2006). The interaction of ROS produced due to redox imbalance and the production of pro-inflammatory cytokines also aggravates hyperglycemia-induced neuroinflammation (Yuan et al. 2019). The structural architecture of the neurons is affected due to the glycosylation of myelin proteins, which impairs its immunogenicity, allowing the entry of monocytes, neutrophils, peripheral blood, and activation of glial cells in the central nervous system. This process triggers the production of pro-inflammatory cytokines and chemokines, increasing neuronal excitability, leading to neuroinflammation and neuronal death. Also, diabetes-induced hypoxia may trigger the elevation of nitric oxide, an important mediator of inflammation processes (Sandireddy et al. 2014). In conclusion, it is evident that neuroinflammation is a consequence of the progression of diabetes. The activation of microglia in specific regions, including disruption of the BBB as well as changes in inflammatory pathways and upregulation of genes associated with pro-inflammatory proteins and markers as well as hyperinsulinemia and chronic hyperglycemia contribute to inflammation of the neurons, which leads to neuronal degeneration and ultimately cognitive decline and impairment.

Mechanistic Insights Involved in Diabetes-Induced Neuroinflammation

Chronic hyperglycemia has been linked with redox imbalance, increased production of free radicals, reduction of blood flow, impaired vascular function, and activation of pro-inflammatory pathways (Giacco and Brownlee 2010; Tangvarasittichai 2015). Consistent high blood glucose triggers the activation of microglia and reduced coverage of pericytes in the BBB, which affects the brain's neuronal architecture (Rom et al. 2019). The disruption of the BBB contributes to neuroinflammation in the brain. Hence, persistent hyperglycemia contributes to neuroinflammation, which triggers neuronal damage and subsequently diabetic encephalopathy. Though the pathological mechanisms involved in hyperglycemia-driven neuroinflammation is multifactorial, one of the major mechanisms involved is the interaction of oxidative stress and inflammatory pathways, which weakens neuronal integrity and aggravates neurodegeneration (Sandireddy et al. 2014). The interaction of these two processes aggravates neuronal dysfunction, which impairs cognitive function and memory. Classical pathways such as the polyol hexosamine, MAPK, and protein kinase C (PKC) and advanced glycation end product (AGEs) are activated due to chronic hyperglycemia. They have been shown to mediate mitochondrial ROS production (Singh et al. 2014). These pathways, alongside impaired redox balance, activate the production of inflammatory markers. High levels of AGEs from lipids and proteins tend to trigger inflammatory mediators, including NF-κB and cytokines (Yan et al. 1994). NF-κB regulates the expression of pro-inflammatory cytokines and has been shown to contribute to the induction of neuronal cell death (Sandireddy et al. 2014). A report showed that oxidative damage to glial cells triggers an increase in pro-inflammatory cytokine levels, which acts on neuronal cell membrane receptors, hence activating neuroinflammation in the brain cells (Myers et al. 2006). The study

of Rom et al. (2019) confirmed the upregulation of pro-inflammatory biomarkers such as IL-9, IL-17A, IL-22, and IL-5 in the cerebral cortex of diabetic rats. Wang et al. (2012) reported that treatment of primary astrocytes from BALB/c mice with high glucose triggered ROS production and activation of NF-kB and signal transducer and activation transcription 3 (STAT-3). The result also showed activation of multiple pro-inflammatory cytokines, which include IL-1β, IL-6, IL-4, and induction of vascular endothelial growth factor (VEGF) expression, which are mediators of neuroinflammation.

Furthermore, amylin accumulation induced by hyperglycemia in diabetic rats can influence neuroinflammation-induced neurological deficits (Srodulski et al. 2014). The progression of diabetes leads to pathological changes in the pancreas involving the accumulation of amylin, which activates inflammatory response that leads to diabetic brain injury and cognitive impairment (Srodulski et al. 2014). Amylin is a peptide with 37 amino acids that have amyloidogenic properties (Kahn et al. 1990). It is produced in the pancreatic islets alongside insulin. Amylin controls peripheral energy balance and secretion of insulin. It also reduces glycogen synthesis and glucose uptake in isolated muscle strips (Westermark et al. 2011; Höppener et al. 1993; Srodulski et al. 2014). However, accumulation of amylin may lead to oligomerization and the formation of toxic amyloid plaques. Previous investigation has shown that hyperamylinemia, which involves over-secretion of amylin, leads to amylin deposition in the pancreas islets, blood vessels, and the brain of diabetic patients (Jackson et al. 2013). The accumulation of amylin deposits has been observed in the blood vessels and perivascular spaces and amylin plaques in the temporal lobe gray matter of diabetic patients (Jackson et al. 2013). Srodulski et al. (2014) also reported that hyperamylinemia induced accumulation of amylin and deposition of oligomerized amylin in rats' brains, thereby inducing an inflammatory response, altering brain structure and causing neurological deficit. The study showed that the accumulation of oligomerized amylin is associated with neuroinflammation and changes in cerebrovascular architecture in rat's brain. The result showed that amylin induced inflammatory response by activating microglia and increasing the phenotypic expression of M1 (IL-1β, IL-6, TNFα, CD74, CD86, NO) and M2 (Arg1, CD36, Mrc1, IL-4Rα, IGF1, IL-10, IL-1rn, TGFβ) markers.

Additionally, beta-amyloid plays a central role in neuroinflammatory mechanisms in brain pathology (Stanciu et al. 2020). A strong association between peripheral changes and brain pathology has revealed the contributory role of increased beta-amyloid levels and hyperglycemia in producing neuroinflammatory biomarkers such as cytokines (Kinney et al. 2018). The accumulation of beta-amyloid leads to high cytokine levels and chemokines in the brain. In a study by Sankar et al. (2020), amyloid-beta and high blood glucose contributed to increase the expression of chemokines and pro-inflammatory cytokines in rats brain (Table 1). This may lead to neuronal degeneration, brain injury, and cognitive impairment. The study also confirms the report that neuroinflammation accompanies amyloid-beta plaque formation (Cai et al. 2018). Experimental evidence also shows that activating p-38 mitogen-activated protein kinase (p-38-MAPK) in the neurons is associated with stress and inflammation (Hsieh et al. 2019). The work of Cai et al. (2018) revealed

Table 1 Summary of mechanisms of neuroinflammatory biomarkers in different diabetic models

Diabetic model	Neuroinflammatory biomarker	Brain region	Reference
Type – 1 rat model	Increase in IL-6 and reduction of BDNF	Hippocampus	Zeinivand et al. (2020)
Type 1 and type 2 rat model	Increased BBB permeability; Upregulation of IL-22, IL-5, IL-9, and IL-17A; increase in microglia reaction and decrease in pericyte coverage	Cortex	Rom et al. (2019)
Ex vivo model	Induction of ROS production and astrocyte activation. Upregulation of TNF-α, IL-1β, IL-4, IL-6; Induction of NF-Kb and STAT 3 activation; Induction of VEGF expression		Primary astrocytes from BALB/C mice Wang et al. (2012)
Type-2 rat model Type 1 and 2 db/db, HFD, STZ, and APP/PS1 models	Hyperamylinemia; upregulation of TNF-α and IL-6; downregulation of IL-10 Upregulation of chemokines (MIP-1α, MIP-1β, and MCP-1) and pro-inflammatory cytokines (L-1α, IFN-γ, and IL-3)		Whole brain Srodulski et al. (2014) Brain cortices Sankar et al. (2020)
STZ-induced diabetic model	Induction of microglial hyperplasia; Increase iNOS levels; Upregulation of NF-kB nuclear transcription		Hippocampus and Frontal cortex Song et al. (2017)
Type-2 diabetic rat model	Impaired GSK activity; Upregulation of COX-2, iNOS, IL-6, NF-kB, and TNF-α		Hippocampus and cortex Datusalia and Sharma (2014)

that high levels of phosphor-p38 MAPK in the brain of APP/PS1/tau rats might be linked to the formation of neurofibrillary tangles and induction of neuroinflammation. Furthermore, MAPKs participate in stress-associated cell activation and inflammation. Moreover, microglial cells exposed to high glucose levels showed the activation of MAPK and PI3K/Akt signaling pathways and impaired NF-kb regulation, which leads to microglia activation and subsequently neuroinflammation (Hsieh et al. 2019) (Table 2).

Another mechanism involving hyperglycemia-driven neuroinflammation can be seen in the upregulation of inflammatory proteins such as HSP-70 and HO-1 as well as an increase in iNOS and COX-2 expression (Hsieh et al. 2019). Upregulation of iNOS and COX-2 expression is associated with pathological stress in neuronal cells, leading to neuroinflammation induced by microglial activation. Oxidative stress and inflammatory responses are induced in the microglia by the activation of these inflammatory proteins via impaired MAPKs, PI3K/AKT, and NF-kB signaling pathways. The cooperation of these signaling cascades contributes to neuroinflammation in neuronal

Table 2 Mechanism of neuroinflammation-driven cognitive dysfunction in different diabetic models

Diabetic model	Mechanism of action	Study	Reference
Type – 1 rat model	Neuroinflammation-induced BBB-dysfunction; deterioration in acquisition abilities and long-term spatial memory; upregulation of LTα (TNFβ) and CD40lg and downregulation of Myd88, which has been identified in Alzheimer's disease and other types of dementia	Y-Maze and Morris Water Maze; immunohistochemical studies	Rom et al. (2019)
Type 1 and 2 rat model	Chronic hyperamylinemia–induced neuroinflammation. Upregulation of M1 (IL-1β, IL-6, TNFα, CD74, CD86, NO) and M2 ((Arg1, CD36, Mrc1, IL4-Rα, IGF1, IL-10, IL-1rn, TGFβ) markers. Decrease in recognition memory, reduced exploratory drive, and impairment in the rotarod test	Animal Behavior; Recognition memory test	Srodulski et al. (2014)
Type 2 diabetic rat model	Impaired GSK-3 activity, upregulation of COX-2, NF-kB; IL-6, and iNOS; impaired modulation of neurotransmitters; cholinergic deficit; altered cognitive skills, memory deficit; learning deficit; behavioral deficit	Behavioral tests; Y-Maze; Morris Water Maze; Passive avoidance Task	Datusalia and Sharma (2014)
Type-1 diabetic model	Upregulation of IL-1β, IL-6, and TNF-α in hippocampus and cortex; learning and memory deficit Neuronal apoptosis; neuroinflammation via upregulation of iNOS, TNF-α, and pNF-kB; memory impairment	Morris Water Maze test. Y-Maze test	Fang et al. (2017)
Type-2 diabetic model	Neuronal apoptosis; neuroinflammation via upregulation of iNOS, TNF-α, and pNF-kB; memory impairment	Morris Water Maze test. Y-Maze test	Rehman et al. (2017)
Type-2 diabetic rat model	Increased acetylcholinesterase activity; cholinergic dysfunction; cognitive decline and memory deficit; neuroinflammation via upregulation of IL-1β, IL-6, TNF-α, and NF-kB	Morris Water Maze test	Chen et al. (2021)

cells. NF-kB mediates the upregulation of HSP-70 while PI3K/AKT activates the increase of HSP-70 and iNOS levels, as reported in the study of Hsieh et al. (2019). However, the MAPKs signaling pathways, including JNK, mediate all the inducible inflammatory proteins including iNOS, HSP-70, and COX-2.

Furthermore, glucose synthase kinase-3 (GSK-3) has been identified to play a role in the induction of cognitive dysfunction by disrupting neurotransmitters'

regulation and inducing neuroinflammation. Hence GSK-3 inhibitors have been identified as a possible therapeutic agent to mitigate diabetes-induced cognitive dysfunction and complications associated with neurological dysfunction. The upregulation of GSK-3 is linked with hyperphosphorylation of tau proteins and formation of microtubules in the brain. Furthermore, GSK-3 plays a major role in the homeostatic control of beta-amyloid peptide and amyloid precursor protein (APP). Hence upregulation of these isoforms may induce aggregation of beta-amyloid protein and formation of amyloid plaques, which in turn trigger over-production of ROS, which attacks brain cells, causing neuronal apoptosis. The result from the study of Datusalia and Sharma (2014) revealed that hyperglycemia reduces the phosphorylation of GSK-3 kinase and induces the neuroinflammatory effects by triggering the production of iNOS, NF-kB, COX-2, IL-6, and the expression of TNF-α in rat's hippocampus and cortex. Previous studies have identified some proinflammatory biomarkers that are associated with neuroinflammation and this include IL-1β, IL-6, IL4-Rα, IL-10, IL-1rn, TNF-α, NF-kB, COX-2, Arg1, CD36, Mrc, CD74, CD86, and NO in specific regions of the brain. The pro-inflammatory markers mediate neuroinflammatory processes in identified regions (cerebral cortex and hippocampus) leading to neurodegeneration, nerve injury, and neuronal death. Moreover, future studies are needed to explore other novel markers involved in neuroinflammation in other regions of the brain and provide more understanding on the biochemical mechanisms.

Cognitive Impairment and Neuroinflammation in Diabetic Animal Models

Cognitive dysfunction and impairment in memory function are complications and consequences of progressive hyperglycemia. Several studies have shown the bidirectional association between insulin resistance and neuroinflammation. Moreover, the relationship between high glucose levels, insulin levels, memory decline, and neuroinflammation has been established. Some findings have suggested that inflammatory responses in different brain regions may contribute to impairment in learning processes and cognitive and memory function (Salkovic-Petrisic et al. 2013; Chu et al. 2014). The progression of diabetes is associated with memory, learning and behavioral problems, and neuroinflammation triggered by hyperglycemia, which has been implicated in the disruptions of neurochemicals associated with cognitive function. Furthermore, in diabetic conditions, inflammatory responses in microglia and inflammatory biomarkers are activated, contributing to neuronal injury and neurodegeneration. The inflammatory responses such as activation of microglia and upregulation of interleukins and chemokines cause neuronal apoptosis, leading to cognitive impairment. Hence, attenuation and/or inhibition of neuroinflammatory responses in the neurons may be a potent therapeutic target to combat diabetic-induced memory impairment. In a type 1 diabetic experimental model, chronic hyperglycemia triggered memory impairment via activation of neuroinflammatory responses and neuronal apoptosis (Fang et al. 2017). Hyperglycemia-induced

cognitive impairment is linked to neuroinflammatory, oxidative injury and neuronal apoptotic mechanisms (Esmaeili et al. 2020). Neuroinflammation-mediated cognitive decline in hyperglycemic rats was attributed to the activation of astrocytes and increase in inflammatory markers (TNF-α, iNOS, and pNF-kB) in the hippocampal region of the brain as well as induction of neuronal apoptosis by increasing C-jun N terminal kinase (p-JNK) activity and disrupting the regulation of synaptic proteins (Rehman et al. 2017). Furthermore, GSK-3 may play a central role in diabetes-induced cognitive dysfunction via induction of neuroinflammation and disruption of neurotransmitters (Datusalia and Sharma 2014).

The disruption in cognitive skills and memory deficit observed in diabetic rats were attributed to neuroinflammatory mechanisms. Experimental investigations suggest the association between impaired memory function and cholinergic dysfunction (Reeta et al. 2017). Choline acetyltransferase and cholinesterase (acetylcholinesterase and butyrylcholinesterase) are important enzymes in the cholinergic system involved in regulating acetylcholine levels (Reeta et al. 2017). Acetylcholine is an important neurotransmitter that aids the transmission of nerve impulses and signals from one neuron to another. The reduction of acetylcholine levels released in cholinergic neurons may lead to progression of cholinergic nerve degeneration, which may hinder neurotransmission of nerve impulse (Tata et al. 2014). Moreover, the degeneration of cholinergic neurons and disruptions in the transmission of nerve impulse due to cholinergic deficit in CNS leads to cognitive decline (Tata et al. 2014). There is a possible correlation between increased activity of acetylcholine regulatory enzymes and activation of pro-inflammatory cytokines (Gatta et al. 2020). Furthermore, the impaired regulation of homeostatic levels of acetylcholine alters the cholinergic function and influences cytokine production, indicating the role of the cholinergic system in neuroinflammation. Hence, acetylcholine plays a central role between the cross talk of the immune system and the CNS and may regulate inflammatory responses; however, imbalance or impaired homeostatic levels of these neurochemicals may contribute to additional pathological inflammatory reactions (Gatta et al. 2020; Di Bari et al. 2016, 2017).

Sivaprakasam (2006) and Wenk et al. (2000) confirmed that neuroinflammatory processes contribute to cholinergic degeneration in the CNS due to reduction of choline acetyltransferase activity, activation of microglial, upregulation of cytokines. Also, neuronal nicotinic acetylcholine receptors commonly expressed in astrocytes and microglia play a major role in neuroinflammation (Sivaprakasam 2006; Piovesana et al. 2021). The study of Chen et al. (2021) revealed that diabetic rats exhibited cognitive decline and memory deficit due to cholinergic deficit aggravated by increased acetylcholinesterase activity and upregulation of neuroinflammatory factors (IL-1β, IL-6, TNF-α, and NF-kB). Impaired GSK-β activity in diabetic rats also triggered cholinergic deficit, depletion of acetylcholine and glutamate levels, and increased gamma-aminobutyric acid in hippocampal and cortex regions of the brain (Datusalia and Sharma 2014). Hence attenuation of cholinergic deficit and inhibition of neuroinflammation could be important therapeutic approaches to mitigate diabetic-driven cognitive deficit, memory loss, and learning and behavioral problems.

Conclusion

DM is a common metabolic disorder that has become a serious public health burden with complications associated with cognitive decline, neuroinflammation, and neuronal degeneration. Most diabetic complications associated with the central nervous systems are mediated via inflammation. Several studies have established that persistent chronic hyperglycemia is associated with cognitive decline, neuroinflammation, and brain injury. Though the pathological mechanisms involved in hyperglycemia-driven neuroinflammation are multifactorial, one of the major mechanisms involved is the interaction between reactive oxygen species produced due to chronic hyperglycemia and pro-inflammatory pathways that activate neuroinflammatory biomarkers leading to disruptions of neuronal integrity, oxidative injury, and neurodegeneration. In the process, classical pathways such as polyol hexosamine, MAPK, and protein kinase C (PKC) and advanced glycation end product (AGEs) are also dysregulated. This may result in mitochondrial ROS production, which interacts with pro-inflammatory markers to induce neuroinflammation in the brain. Some of the biomarkers that have been identified in the cortex and hippocampal regions of the brain include IL-1β, IL-6, IL4-Rα, IL-10, IL-1rn, TNF-α, NF-kB, COX-2, Arg1, CD36, Mrc, CD74, CD86, and NO. Another biochemical mechanism of hyperglycemia-driven neuroinflammation involves the accumulation of amylin, which oligomerizes and form toxic plaques in the brain. These plaques can induce ROS production, activate microglia, and upregulate M1 and M2 inflammatory markers. The upregulation of inflammatory proteins such as iNOS, HSP-70, and GSK-3 isoforms may trigger microglial activation and mediate the activation of ROS and neuroinflammatory signals via impaired MAPK, JNK, and PI3K/AKT pathways. The triggered neuroinflammatory signals and processes activated contribute to cognitive dysfunction, memory and behavioral problems due to impaired regulation of neurotransmitters, disruption of neurotransmission and neurochemicals associated with memory function. The highlighted biochemical mechanisms of diabetes-driven neuroinflammation and cognitive decline could be important therapeutic strategies to mitigate diabetic complications associated with neurological deficit and memory problems. Future studies may explore more neuroinflammatory processes involved in diabetic-induced cognitive decline to identify new biomarkers associated with neuroinflammation. This will provide more insights and understanding for the possible treatment of diabetic complications associated with neuroinflammation and memory problems (Fig. 1).

Application to Prognosis, Other Diseases, or Conditions

In this chapter, some neuroinflammatory biomarkers have been highlighted, which revealed the associations between diabetes and neuronal damage as well as cognitive problems. Neuroinflammatory pathways are activated due to mitochondria dysfunction and elevated production of reactive oxygen species, which leads to upregulation

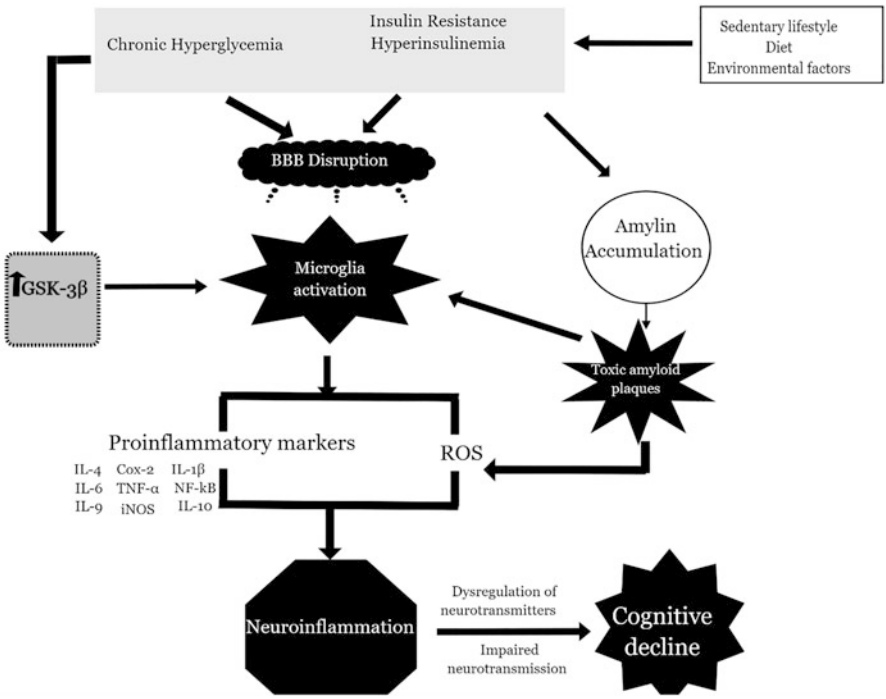

Fig. 1 Biochemical mechanisms of Diabetes-induced neuroinflammation and cognitive decline

of pro-inflammatory cytokines and other inflammatory biomarkers causing neuroinflammation and subsequently neurodegeneration. Furthermore, the activation of inflammatory responses and induction of neurodegeneration hinders neurotransmission or nerve impulses, which impairs cognitive function, learning processes, and behavior. Experimental evidence shows that cognitive impairment has been correlated with cholinergic dysfunction and neuroinflammatory biomarkers such as IL-1β, IL-6, TNF-α, and NF-kB. Furthermore, studies have shown that hyperglycemia-driven neuroinflammation may lead to vascular dementia and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Madhusudhanan et al. 2020; Sergi et al. 2019; Hassan et al. 2020). Hence, one of the important therapeutic strategies to manage these neurodegenerative diseases associated with diabetes may involve attenuation of neuroinflammation by targeting neuroinflammatory and oxidative stress biomarkers.

Key Facts

- The prevalence of diabetes is increasing due to several factors, and its progression may trigger other degenerative diseases.

- Neuronal damage, triggered by hyperglycemia-driven neuroinflammation is one of the end-organ damage and complication of the progression of diabetes.
- Activated neuroinflammatory pathways triggered by persistent hyperglycemia is a key contributing factor to neurodegeneration and loss of memory function in the brain.
- Elevated levels of neuroinflammatory biomarkers in different parts of the brain may help to identify neuroinflammation and possible memory problems in diabetic patients.
- Neuroinflammatory biomarkers are major therapeutic targets that can be explored to mitigate neuronal degeneration and cognitive dysfunction in chronic diabetic patients.

Mini Dictionary

Cognitive dysfunction: A mental health problem that is associated with impairment in learning, memory, perception and problem solving.

Hyperglycemia: A condition involving excessive levels of glucose in the blood.

Neurodegeneration: The progressive deterioration of the neuronal architecture in the brain, which leads to neuronal injury, damage, and cell death.

Neuroinflammation: A pathophysiological mechanism, which contributes to neurodegeneration via activation of inflammatory response in the central nervous system.

Oxidative stress: The imbalance between systemic levels of reactive oxygen species levels produced in cells and tissues and the antioxidant defense mechanism required to detoxify these reactive products.

Summary Points

- Diabetes and its progression are linked to neurodegeneration via disruption of the blood–brain barrier, induction of oxidative stress, and activation of neuroinflammatory pathways.
- Persistent hyperglycemia is a risk factor for progressive degeneration of the peripheral and central nervous system.
- Pro-inflammatory cytokines activate neuroinflammatory pathways and elevation of neuroinflammatory biomarkers, which contribute to cerebrovascular pathology, cognitive impairment, and behavioral problems.
- Neuroinflammatory responses induced by persistent high blood glucose may contribute to loss of neurotransmission and impairment in cognitive function.
- Other neuroinflammatory pathological mechanisms induced by chronic hyperglycemia are yet to be explored.
- The management of some neurodegenerative diseases such as Alzheimer's and Parkinson's diseases associated with diabetes could be via attenuation of neuroinflammation by preventing and inhibiting mitochondrial dysfunction, ROS production, and upregulation of neuroinflammatory biomarkers.

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Part VI

Diet and Diabetes

Selenium and Risk of Diabetes

52

Shinje Moon and Chang-Myung Oh

Contents

Introduction 1076
Association Between Selenium and the Risk of T2DM 1077
Recent Updates on Clinical Studies 1077
Selenium and Gestational Diabetes 1078
Selenium and Diabetes Complications 1079
Potential Mechanisms of Action of Selenium on Glucose Homeostasis 1080
Conclusion 1081
Summary Points 1082
References 1082

Abstract

Selenium is mainly involved in the synthesis of selenoproteins in living organisms, and its primary role is to reduce oxidative stress in the body. Antioxidative properties of selenium led to the expectation that selenium would be protective against type 2 diabetes (T2DM). However, findings from recent epidemiological studies have raised concerns that high selenium levels in the body may be associated with T2DM or insulin resistance. Hence, the chapter goes on to discuss the current understanding of the role of selenium on glucose homeostasis and the association between selenium exposure and the risk of T2DM.

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Keywords

Selenium · Selenoprotein · Diabetes mellitus · Metabolic syndrome · Insulin resistance

Abbreviations

GDM	Gestational diabetes mellitus
GPX	Glutathione peroxidase
MetS	Metabolic syndrome
SeP	Selenoprotein P
T2DM	Type 2 diabetes
TXNRD	Thioredoxin reductase

Introduction

Selenium is an essential nutrient for humans and plays important roles in reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection in the form of various selenoproteins (Sunde 2012). Selenium mainly exists in the form of selenocysteine or selenomethionine in animal and plant tissues and in the form of selenoprotein P (SeP) or glutathione peroxidase (GPX), a selenoprotein with selenocysteine residues, in the plasma. Selenium is present as a micronutrient in many foods or can be consumed as a dietary supplement. Dietary selenium is supplied in two forms, inorganic (selenate and selenite) and organic (selenomethionine and selenocysteine) (Sunde 2006; Terry 2012). Selenium intake varies greatly depending on the dietary pattern of each country and the selenium content in the soil in which particular food is produced. In the United States and United Kingdom, meat and bread are the main sources of selenium (Rayman 2012). In Japan, fish and shellfish account for approximately 50% of selenium (Yoneyama et al. 2008), and in Korea, grains are known to be the main source of selenium (Choi et al. 2009).

Selenium is mainly involved in the synthesis of selenoproteins in living organisms, and its main role is to reduce oxidative stress in the body. Selenium is involved in antioxidant functions, redox regulation, thyroid function, and fertility through 35 different selenoproteins. Representative selenoproteins include SeP, selenoprotein W, GPX, thioredoxin reductase (TXNRD), and iodothyronine deiodinase, which regulates thyroid hormone metabolism (Berry and Larsen 1992), and the main form of selenium that exhibits biological functions in the body is selenocysteine. Selenoproteins have one or more selenocysteine residues in their primary structure (Zachara 1992; Brown and Arthur 2001). GPX acts as an antioxidant by decomposing hydrogen peroxide in the body to prevent cell damage (Flohe 1988). TXNRD is also involved in the regeneration of the antioxidant system by reducing thioredoxin and plays an important role in early stages of cell differentiation (Brown and Arthur 2001). As a glycoprotein, SeP is the most abundant

selenoprotein in the plasma and plays a role in transporting and storing selenium. SeP is also found in cell membranes and is presumed to have an antioxidant function (Zachara 1992; Brown and Arthur 2001; Hill and Burk 1997; Burk et al. 1995). Thus, selenium-containing proteins are involved in various metabolic and physiological functions, including antioxidant defense, reproduction, muscle development and function, thyroid hormone metabolism, and immune responses.

Because of the antioxidant properties of some selenoproteins, it was assumed that selenium would be protective against type 2 diabetes (T2DM). Several in vivo and in vitro studies have found that selenium has antidiabetic and insulin-mimicking properties. Several epidemiological studies, however, have raised concerns that high selenium levels in the body may be linked to T2DM or insulin resistance. Hence this chapter goes on to discuss current understanding of the role of selenium on glucose homeostasis and the association between selenium exposure and the risk of T2DM.

Association Between Selenium and the Risk of T2DM

Recent Updates on Clinical Studies

T2DM is characterized by varying degrees of peripheral insulin resistance and defects in insulin secretion. Although the mechanisms that underlie insulin resistance and T2DM are not fully understood, several studies have suggested a role for oxidative stress in the onset and progression of T2DM (Evans et al. 2003; Pérez-Matute et al. 2009). Selenium has been assumed to help in the prevention and therapy of T2DM because of its antioxidative properties (Kljai and Runje 2001; Faure 2003; Rajpathak et al. 2005). However, Stranges et al. (2007) conducted a secondary analysis of the Nutritional Prevention of Cancer trial and found an excess risk of T2DM among subjects receiving selenium supplementation (200 μg/day) compared with those receiving a placebo (hazard ratio [HR] = 1.55, $p = 0.03$), which generated a concern. By contrast, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), the largest prostate cancer prevention trial with 35,533 participants, showed a nonsignificant increase in the risk of T2DM (relative risk [RR] = 1.07, $p = 0.16$) after supplementation with 200 μg/day of selenium compared with that of a placebo (Lippman et al. 2009). The Selenium and Celecoxib Trial evaluated the effects of selenium (200 μg/day) and celecoxib (400 mg/day) on the prevention of colorectal adenoma. Although the overall risk of T2DM was not significantly increased, a subgroup analysis showed a significantly increased risk for T2DM in older participants (>63 years of age) receiving selenium compared with that in the placebo group (HR = 2.21, $p = 0.03$) (Thompson et al. 2016). A recent meta-analysis of five randomized controlled trials (RCTs) also showed a significantly increased risk of T2DM in participants receiving selenium compared with that in the comparison groups (RR = 1.11, 95% CI: 1.01–1.22) (Vinceti et al. 2018). However, all the trials were conducted using organic selenium or selenized yeast (Vinceti et al. 2017a, 2018). Considering a higher toxicity of inorganic species of the element compared with the organic species, the lack of evaluation of the effects of

inorganic selenium on the risk of T2DM was a limitation of the previous RCTs (Oliveira et al. 2017). Since most RCTs were conducted on US populations, additional research considering differences in the race, soil selenium concentrations, and regional dietary habits is necessary.

Several observational studies have been conducted to investigate the relationship between selenium levels and the risk of T2DM. High serum selenium levels were associated with the prevalence of T2DM in the NHANES III and NHANES 2004–2005 studies (Bleys et al. 2007; Laclaustra et al. 2009). An updated study using NHANES 2011–2014 data found a dose-dependent positive relationship between serum selenium levels and the prevalence of T2DM (Moon et al. 2019). A recent meta-analysis of 34 studies has shown that selenium exposure across a wide range of concentrations, particularly a blood selenium level above 120 μg/L and dietary selenium intake of 80 μg/day, increases the risk of T2DM (Vinceti et al. 2021). In this meta-analysis, dietary selenium intake and urinary selenium concentrations were found to have linear associations with the risk of T2DM, while blood selenium concentrations had a J-shaped relationship with the risk of T2DM (Vinceti et al. 2021).

Selenium and Gestational Diabetes

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance that begins or is discovered during pregnancy (Metzger 1998). Selenium, as an essential element in the human body, has shown significant associations with GDM. Recently, Xu et al. (2022) have performed a meta-analysis to investigate the clinical relationship between serum selenium levels and GDM. This meta-analysis included 27 studies and indicated that serum selenium levels in patients with GDM were significantly lower than those in healthy controls (standard mean difference $= -1.29$; 95% CI: -1.60 to -0.97, $p < 0.00001$). These data suggest that there are significant associations between low serum selenium levels and GDM.

Based on these significant associations, Moshfeghy et al. (2020) proposed the use of the serum selenium level as a predictive value for GDM diagnosis. In this nested case–control study, the serum selenium levels in patients with GDM were lower than those in healthy pregnant controls, and the cutoff level was 48.2 μg/L in the first trimester (sensitivity 83.3% and specificity 94%) (Moshfeghy et al. 2020).

There has been no observational study to establish the causality between serum selenium and GDM. A recent RCT has investigated the effects of selenium supplementation on GDM (Najib et al. 2020). In this study, supplementation with 100 μg of selenium for 12 weeks did not improve glucose homeostasis in patients with GDM (Najib et al. 2020). However, in another study, supplementation with 200 μg of selenium for 6 weeks reduced the fasting plasma glucose and serum insulin levels and improved other parameters related to glucose homeostasis (Asemi et al. 2015). Further large, well-designed studies are needed to clarify the effects of selenium on GDM.

Genetic studies have provided evidence on the relationship between selenium and GDM. Among SNPs in the SeP1 gene (SEPP1), rs3877899 was significantly associated with the maintenance of selenium levels during pregnancy (Mao et al. 2016) and with GDM etiopathogenesis (Degirmencioglu et al. 2018).

Selenium and Diabetes Complications

Selenium has shown protective effects against diabetic liver injury. In an alloxan-induced diabetic rat model, selenium supplementation in the form of sodium selenite significantly decreased aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, and insulin levels in the blood (Zou et al. 2016). Compared with those in nontreated diabetic rats, the histologic features of diabetic injury, such as sinusoidal congestion, lipid accumulation, and centrilobular hepatocyte degeneration, were all improved in selenium-treated diabetic rats (Zou et al. 2016).

Selenium also plays protective roles in the diabetic heart. As an antioxidant, selenium suppressed oxidative stress-induced deleterious signals and reduced cardiac hypertrophy in diabetic rats (Dhanya et al. 2014; Okatan et al. 2013; Mushtaq et al. 2021). In an STZ-induced diabetic rat model, selenium reduced cardiomyocyte apoptosis by modulating the Toll-like receptor 4 (TLR4) pathway (Haitao et al. 2013).

A population-based cohort study (NHANES) discovered that higher selenium levels were associated with lower all-cause and heart disease mortality among people with type 2 diabetes (Qiu et al. 2022). Intriguingly, selenium did not show significant associations with coronary artery disease (CAD). Serum selenium levels were not different between people with and without diabetes (Sotiropoulos et al. 2011). A recent Mendelian randomization study has also found little association between selenium levels and CAD (Rath et al. 2021).

Selenium also has beneficial effects against the progression of diabetic nephropathy. Selenium supplementation suppressed the levels of inflammation- and oxidative stress-related biomarkers, such as matrix metalloproteinase 2 (MMP-2) and plasma nitric oxide, and increased total plasma antioxidant capacity in patients with diabetic nephropathy (Bahmani et al. 2016). In an STZ-induced diabetic rat model, selenium nanoparticles reduced the expression of profibrotic markers, such as TGF-β1, desmin, and vimentin, improved the antioxidant capacity, and blocked renal morphological alterations (Khan and Sheppard 2006). A selenium-deficient diet increased renal oxidative stress and TGF-β1 signaling in a diabetic rat model (Reddi and Bollineni 2001).

Plasma selenium and GPX levels were shown to be significantly lower in diabetic patients with macroalbuminuria than in diabetic patients with or without microalbuminuria, and in healthy controls (Sedighi et al. 2014). In line with these results, selenium supplementation for 12 weeks to patients with diabetes and microalbuminuria did not show beneficial effects (Ghadiri-Anari et al. 2020).

Selenium shows beneficial effects against nerve injury. Thus, selenium improved nerve regeneration in a sciatic nerve crush model and reduced sciatic nerve damage

due to ischemia/reperfusion injury by decreasing TNF-α signaling (Dolkhani et al. 2020). Diphenyl diselenide, an organic selenium compound, significantly suppressed glucose-induced cytotoxicity and oxidative stress in a Schwann cell line and improved the nerve conduction velocity in an STZ-induced rat model by activating the NRF2/KEAP1 pathway (Wang et al. 2020). Furthermore, as an antioxidant, selenium protects retinal pigment epithelial cells from glucose-induced oxidative stress (González de Vega et al. 2018) and improves diabetic wound healing by increasing angiogenesis (Bajpai et al. 2011).

Potential Mechanisms of Action of Selenium on Glucose Homeostasis

Recent studies have indicated that selenium plays a role in the insulin-signaling cascade in pancreatic beta cells and other peripheral organs. Evidence suggests that adequate selenium concentrations are required for insulin secretion and action (Fontenelle et al. 2018). In the mouse beta cell line Min6, selenium treatment upregulated insulin promoter factor 1 (Ipf1) and insulin gene expression and stimulated insulin secretion (Campbell et al. 2008). In a streptozotocin (STZ)-induced diabetic rat model, selenium supplementation increased glucagon-like peptide 1 (GLP1) signaling, the numbers of islets and viable beta cells, and preproinsulin expression in the pancreas (Barakat et al. 2016).

Insulin secretion is also affected by selenoproteins. In Min6 cells, the overexpression of mouse selenoprotein K increased while its knockdown decreased the insulin secretion by changing free cytosolic Ca2+ release from the endoplasmic reticulum (Meng et al. 2017). By contrast, SeP suppresses insulin secretion. In a mouse model of type 2 diabetes, excess SeP administration decreased high glucose-induced insulin secretion, but this effect was reversed by the administration of a neutralizing antibody against SeP (Mita et al. 2017).

Selenium also plays a role in the development of peripheral insulin resistance (Fontenelle et al. 2018). According to a recent meta-analysis, selenium levels in the blood/serum were higher in subjects with metabolic syndrome (MetS) than in healthy controls (Tinkov et al. 2020). At the same time, higher SeP levels were associated with a lower risk of MetS (OR = 0.995; 95% confidence interval [CI]: 0.989–1.00, $p = 0.04$); furthermore, SeP levels were inversely associated with the waist circumference (OR = 0.995; 95% CI: 0.990–1.00) (Gharipour et al. 2017). By contrast, a study of Korean adults without diabetes found that higher SeP levels were associated with both visceral obesity and NAFLD (Choi et al. 2013). Furthermore, circulating SeP levels correlated with the HOMA-IR index and were more than threefold higher in obese than in nonobese patients (Choi et al. 2013).

In the liver, selenium modulates both gluconeogenesis and glycolysis. In the rat liver, selenium administration was shown to decrease the levels of pyruvate carboxylase and glucose-6-phosphatase while increasing those of glucokinase and phosphofructokinase (Iizuka et al. 1993). In diabetic rats, selenium normalized the altered activities of glucose-metabolizing enzymes and reduced glucose uptake and utilization in the liver (Chen et al. 2015). In a mouse model of high-fat diet (HFD)-induced

fatty liver disease, selenium showed protective effects against hepatic injury and insulin resistance by alleviating oxidative stress (Wang et al. 2022). By contrast, several other animal studies have reported that selenium can increase insulin resistance in the liver. In rats, the supplementation with high doses of selenium increased lipolysis in the adipose tissue and free fatty acid accumulation in the liver (Wang et al. 2014).

Furthermore, selenium improves the physiological functions of the skeletal muscle. In a mouse study, selenium supplementation increased the speed of voluntary running and the daily distance covered by augmenting calcium release from the sarcoplasmic reticulum (Bodnár et al. 2016). In a diabetic mouse model, selenium-enriched exopolysaccharide supplementation improved glucose uptake and utilization in skeletal muscles through the AMPKα2/PGC-1α pathway (Zhou et al. 2014). On the other hand, selenium compounds, such as selenite and methylseleninic acid, delayed insulin signal transduction in rat L6 myotubes (Pinto et al. 2011).

In adipose tissues, selenium and selenoproteins regulate adipocyte development and functioning (Tinkov et al. 2020). Selenium supplementation in the physiological range was shown to activate 3 T3-L1 preadipocyte differentiation by increasing cyclin-dependent kinase (CDK1 and CDK2) expression (Tinkov et al. 2020). SeP is upregulated in the liver of patients with type 2 diabetes and worsens glucose intolerance (Mita et al. 2017; Misu et al. 2010). Antioxidant selenoproteins, such as GPXs and TXNRDs, play roles in the endocrine system and reduce oxidative stress (Tinkov et al. 2020). Gpx1 knockout mice fed on HFD had significantly lower levels of hepatic steatosis, insulin resistance, and resistance to diet-induced obesity than wild-type mice (Merry et al. 2014). TXNRD transcription in human SAT is linked to lipogenesis and insulin resistance in adipocytes (di Giuseppe et al. 2019). Clinical studies have also found a link among TXNRD expression/activity in adipose tissue, antioxidant regulatory pathways, and excessive adiposity (Tinkov et al. 2020).

Conclusion

Although selenium improves insulin secretion by pancreatic beta cells in animal and human studies, clinical studies have shown opposite effects of selenium supplementation in patients with diabetes. A recent Mendelian randomization analysis using genome-wide association studies has found a genetically predicted association of selenium with a higher risk of type 2 diabetes (OR = 1.27; 95% CI: 1.07–1.50) (Rath et al. 2021). This result implies that selenium may increase the risk of type 2 diabetes by increasing insulin resistance, despite improving pancreatic beta cell function.

Intriguingly, selenium also shows inconsistent results in terms of its effects on insulin resistance. A recent population-based cohort study (NHANES) has reported that a 10 μg/L increase in the level of selenium was associated with a 1.5% increase in insulin resistance and a 1.7% increase in HOMA-IR (Cardoso et al. 2021). By contrast, dietary selenium intake was negatively correlated with HOMA-IR in another cohort (Newfoundland population) (Wang et al. 2017). In the Newfoundland

population, a higher dietary selenium intake was associated with lower insulin resistance when the total dietary selenium intake was less than 1.6 g/kg/day; above this point, the beneficial effect of selenium was lost (Wang et al. 2017).

These data imply that the inconsistencies between studies might be due to U-shaped metabolic effects of selenium. As selenium is an antioxidant and essential mineral, its deficiency leads to a wide range of cellular dysfunctions and causes critical diseases, such as cardiovascular disease, infertility, and cognitive decline (Shreenath et al. 2022). Animal studies have reported that selenium deficiency resulted in the atrophy of the pancreas in chickens, impaired islet functions in rats, and diabetes and MetS phenotypes in mice (Zhou et al. 2013). Excessive selenium also induces many disorders in the body. At high concentrations, selenium acts as a prooxidant and induces reactive oxidative stress (Wallenberg et al. 2014; Lee and Jeong 2012). This toxicity may be the cause of increased insulin resistance and the positive associations between selenium levels and diabetes (Cardoso et al. 2021; Vinceti et al. 2018; Kim et al. 2019).

This U-shaped metabolic effect implies that further clinical and preclinical studies are needed to find both normal range of selenium levels in blood and daily requirements. Current recommended dietary selenium allowance for adult over 19 years old is 55 μg (0.70 μmol) daily. And tolerable upper intake level in adult is 400 μg (0.70 μmol) (Monsen 2000). But this tolerability is based on the result of acute toxicity (selenium poisoning) and chronic toxicity (selenosis) of selenium intake such as hair loss, gastrointestinal disturbance, skin rash, and nervous system abnormalities (Monsen 2000). More research into metabolic dysfunctions as a side effect of selenium intake is needed to determine the optimal range of selenium levels and daily intake.

Summary Points

- Selenium is mainly involved in the synthesis of selenoproteins in living organisms, and its primary role is to reduce oxidative stress in the body.
- Antioxidative properties of selenium led to the expectation that selenium would be protective against type 2 diabetes (T2DM).
- Findings from recent epidemiological studies have raised concerns that high selenium levels in the body may be associated with T2DM or insulin resistance.
- This chapter goes on to discuss the current understanding of the role of selenium on glucose homeostasis and the association between selenium exposure and the risk of T2DM.

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Part VII

Models and Modeling in Diabetes

Models of Diabetes in Rats: A Focus on Diabetic Neuropathy and Biomarkers

53

Che Aishah Nazariah Ismail and Idris Long

Contents

Introduction 1091
Rat Model of Diabetic Neuropathy 1092
Biomarkers of Diabetic Neuropathy in Rat Models 1093
Oxidative Stress Markers 1093
Immune Cells 1096
Cell Receptors 1101
Enzyme-Related Markers 1103
Other Protein 1105
Conclusion 1106
Applications to Prognosis, Other Diseases or Conditions 1106
Mini-dictionary of Terms 1106
Key Facts 1106
Key Facts of Diabetic Neuropathy 1106
Key Facts of Rat Model of Diabetic Neuropathy 1107
Summary Points 1107
References 1107

Abstract

The increased prevalence of diabetic neuropathy (DN), as reported by World Health Organization, is seriously alarming. There is an unmet need to identify and understand the roles of potential biomarkers for the diagnosis and new therapeutic discovery to combat the disease progression. In this chapter, several key players

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leading to alterations during the pathophysiology of DN will be discussed with regard to the discoveries in the established rat models. These biomarkers include certain glial cells, receptors, enzymes, and inflammatory and oxidative stress markers that play significant functions in facilitating disease progression. This review will hopefully provide important insights and understanding regarding the mechanisms involved, thereby a number of therapeutics, either modern or alternative medications, can be effectively developed.

Keywords

Diabetic neuropathy · Rat model · Glial cells · *N*-methyl-D-aspartate receptor · Reactive oxygen species · Caveolin-1 · Adiponectin · Gamma-amino butyric acid receptor · Nuclear factor-kappa B · Heme oxygenase-1 · Interleukin-1β · Tumor necrosis factor-α · Monocyte chemoattractant protein-1 · NADPH oxidase · Transient receptor potential vanilloid-1

Abbreviations

AGEs	Advanced glycation end-products
AR	Aldose reductase
ATP	Adenosine triphosphate
Bcl-3	B-cell lymphoma-3
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium ion
CAV-1	Caveolin-1
Cl^{-}	Chloride ion
DM	Diabetes mellitus
DN	Diabetic neuropathy
DNA	Deoxyribonucleic acid
DREAM	Downstream regulatory element antagonist modulator
DRG	Dorsal root ganglia
ERK/pCREB	Extracellular signal-regulated kinases/phosphorylated cAMP response element binding protein
GABA	Gamma-amino butyric acid
GFAP	Glial fibrillary acidic protein
GluN1	N1 subunit of N-methyl-D-aspartate receptors
GR	Glutathione reductase
GSH	Glutathione
GSHPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
ICAM-1	Intracellular adhesion molecule-1
IKK	IκB kinase
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6

JAK2/STAT3	Janus kinase-2/signal transducer and activator of transcription-3
JNK	c-Jun-N-terminal kinase
KCC2	Potassium-chloride transporter
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
Mg^{2+}	Magnesium ion
MWT	Mechanical withdrawal threshold
NAD^{+}	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide with hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NCV	Nerve conduction velocity
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells or nuclear factor kappa B
NLRP3	Nod-like receptor protein-3
NMDARs	*N*-methyl-D-aspartate receptors
NOX	NADPH oxidase
NR2B	NR2B subunit of *N*-methyl-D-aspartate receptors
O_2^-	Superoxide anion
OLETF	Otsuka Long-Evans Tokushima Fatty
p38 MAPK	p38 mitogen-activated protein kinase
PPAR-ɣ	Peroxisome proliferator activated receptor-gamma
PWT	Paw withdrawal threshold
RAGE	Receptor for advanced glycation end-product
RDD	Rate of depression
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
SDT	Spontaneously diabetic tori
SGCs	Satellite glial cells
SOD	Superoxide dismutase
STZ	Streptozotocin
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TrkB	Tyrosine kinase receptor
TRPV1	Transient receptor potential vanilloid-1
TWL	Thermal withdrawal latency
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Introduction

Diabetic neuropathy, or synonymously known as peripheral diabetic neuropathy (DN), is a late complication of type I or II diabetes mellitus (DM), which is also the leading cause of foot ulcers and limb amputation (World Health Organization

2020). Patients suffering from DN have severe healthcare burden and frequently complained of poor life quality. Recently, the prevalence of DN reported in type I and II DM was 40.3% and 29.1%, respectively, with increased prevalence in patients of more than 70 years old (47.7–57.5%) (Pfannkuche et al. 2020). World Health Organization (WHO) reported the alarming prevalence of 5% DM within 6 years, with half of the death cases attributed to patients below 70 years of age. It is asserted that the root cause of mortality from DN is poor glycemic control or improper control of DM (Islam 2013; Pfannkuche et al. 2020). Glycemic management could prevent neuropathy in individuals with type I DM, but not type II DM since obesity constitutes a higher risk factor for developing neuropathy. Notably, DN involves nerve injuries with the longest nerves innervating toes and extending proximally. The damage to these proximal nerves results in numbness, tingling sensations, pain, and/or weakness that begin at the distal lower extremities (Callaghan et al. 2020). There are also abnormal pain sensations, including paraesthesia, hyperalgesia, allodynia, and spontaneous pain, that usually coexist with the impaired sensory functions (Calcutt 2002; Islam 2013).

Rat Model of Diabetic Neuropathy

The establishment of the diabetic animal model has hugely facilitated researchers in exploring the abnormal pathophysiology of diabetic-induced neuronal injury since there are limitations for the study on human subjects and sample collection. For decades, an animal model of diabetes has been established to mimic the pathogenesis of DN, resembling the complications in humans. The majority of the diabetic rat model is conventionally developed rather than obtained authentically due to several restrictions. In patients, the classification of DN is based on the types of injured nerves, either sensorimotor (longer nerve fiber neuropathy) or autonomic (heart, lung or blood vessel neuropathy) (Adki and Kulkarni 2020). However, these specific types of DN are not emphasized in the animal model due to several limitations. Generally, the DN resulting from either type I or II DM can be developed in the rat model. The DN from type I DM can be induced by injecting a single high dose of chemical like streptozotocin (STZ) intraperitoneally (Afrazi and Esmaeili-Mahani 2014; Ismail et al. 2018a, 2020; Zhou et al. 2018) while DN from type II DM is developed by combining a high-fat diet and lower concentration of STZ for a certain period (Eissa et al. 2017; Ji et al. 2017; Chen et al. 2020). Besides that, Ismail and colleagues have determined two variants, either painful or painless DN in the rat model which showed distinguishable modifications in behavioral responses and nociceptive biomarkers at the spinal cord level (Ismail et al. 2018a, 2020). Nonetheless, the majority of previous studies investigated the mechanisms involved in the painful variant of the DN model (Fox et al. 1999; Morrow 2004; Li et al. 2019; Djouhri et al. 2020; Lee-Kubli et al. 2021) rather than the painless DN.

In previous literature, several protocols have been introduced to develop the DN rat model, including alloxan- or streptozotocin (STZ)-induced rat model, spontaneously diabetic WBN/Kob rat model, Otsuka Long-Evans Tokushima Fatty (OLETF)

rat model, surgically induced neuropathic rat model, and genetically modified spontaneously diabetic torii (SDT) fatty rat model. However, the most developed DN rat is through the STZ injection at various dosages (Islam 2013). The developed DN rat model from any protocols, however, should comply with the best criteria as listed by Islam (2013); the rat model should (1) exhibit all major pathogenesis of DN or painful DN with other minor pathophysiology that is usually found in DN patients, (2) have a high sensitivity to anti-diabetic or anti-neuropathic medications, and (3) be eligible for studying the pathophysiology of DN and for the routine pharmacological screening of antidiabetic or anti-neuropathic medications. It is not easy to completely mimic the human DN since the experimental differences such as animal strain, types of diabetes, method of diabetic induction, duration of diabetes, animal age, and gender exists (Feldman et al. 2008; Islam 2013). However, the advantages of developing the DN rat model are the pathogenesis of diabetic complications that appeared in humans, whether at the early or late stages, can be mimicked, and it is affordable given the limited research supply (Baig and Panchal 2019).

Multiple metabolic factors have been speculated as the culprit of diabetic-associated neuropathy besides hyperglycemia. Indeed, the pathogenesis of DN is very complex involving the combined synergistic effects of certain biomarkers such as oxidative stress, production of advanced glycation end products (AGEs), diminution of nitric oxide level, imbalanced protein kinase activities, and reduced neurotrophic peptide factors that eventually contribute to the damage of distal nerves and loss of sensation (Dewanjee et al. 2018). For instance, dyslipidemia and modified sphingolipid metabolism emerge as the novel mainstream to nerve injury in type II DN that negatively affect several cells in the peripheral nervous system including neuronal axons, dorsal root ganglia (DRG), and Schwann cells (Callaghan et al. 2020). These effects cause further consequences involving the formation of excessive reactive oxygen species (ROS), loss of adenosine triphosphate (ATP) production, mitochondrial dysfunction, activation of stress pathways, and glycation of important proteins to form AGEs. Besides that, amplification of glucose auto-oxidation, AGE production, and activation of the polyol pathway also occur (Gugliucci 2017). The excessive level of oxidative stress in the cells resulted from the uncontrolled hyperglycemia leads to endoplasmic reticulum stress, deoxyribonucleic acid (DNA) damage, apoptosis, pro-inflammatory signaling activations and mechanisms that eventually contribute to nerve damage (Yagihashi et al. 2011; Kandhare et al. 2012; Callaghan et al. 2020). In this chapter, the roles and contributions of several important biomarkers in the pathophysiology of DN will be discussed as discovered in various DN rat models to give crucial insights regarding the pathomechanisms involved.

Biomarkers of Diabetic Neuropathy in Rat Models

Oxidative Stress Markers

Oxidative stress has been shown to contribute to the pathogenesis of DN. It starts with the occurrence of persistent hyperglycemia, which causes glucose auto-oxidation in various critical cells and tissues due to a failure to detoxify free radicals

produced during metabolic activities (Adki and Kulkarni 2020). In turn, this activity triggers the polyol pathway, which turns the excess glucose into sorbitol before being converted to fructose via the action of sorbitol dehydrogenase. As a result, these biological systems directly affect the formation of nicotinamide adenine dinucleotide phosphate (NADPH). The depleted formation of NADPH leads to reduced production of glutathione (GSH), a primary endogenous antioxidant that terminally leads to the hyperproduction of oxidative stress and impairment of nerve conduction (Várkonyi et al. 2017; Adki and Kulkarni 2020). The upregulated oxidative stress markers are one of the early phenomena detected in the development of insulin resistance, inflammation, and modification of the aldose-reductase pathway (Yu et al. 2012; Adki and Kulkarni 2020) (Fig. 1).

Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide are the essential forms of free radicals released in the body during normal physiology. Its production is strictly controlled in healthy cells, but its hyper release may contribute to cellular damage during metabolic dysfunction. The O_2^- is formed by the mitochondrial electron transfer chain during the oxidation process of nicotinamide adenine dinucleotide (NADH) to the oxidized nicotinamide adenine dinucleotide (NAD^+) and oxidative enzyme by-products (Vincent et al. 2004). Overproduction of O_2^- in β-cell may activate stress-signaling mechanisms to stimulate downstream effectors, including nuclear factor kappa-light-chain-enhancer of activated B cells

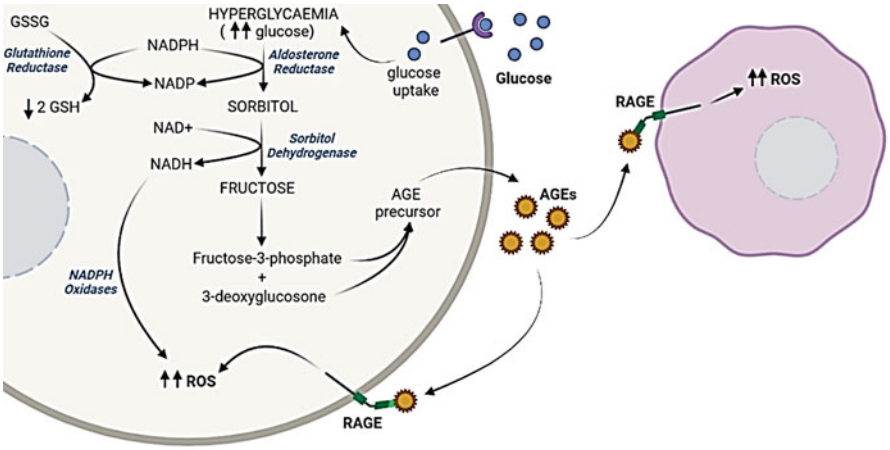

Fig. 1 The production of reactive oxygen species (ROS) following the prolonged hyperglycemia during diabetic neuropathy. Aldose reductase (AR) converts the increased glucose level in the cell to sorbitol which is further fragmented into fructose by sorbitol dehydrogenase enzyme. At the same time, AR competes the NADPH with glutathione reductase (GR) causing the depleted level of glutathione (GSH). The productions of fructose byproducts; fructose-3-phosphate and 3-deoxyglucosone contribute to the higher production of advanced glycation end-products (AGEs). The binding of AGEs to its receptor (RAGE) leads to the increased formation of ROS in the same or other cells. Hyper formation of ROS is also occurred from the formation of nicotinamide adenine dinucleotide (NADH) by NADPH oxidase (NOX). (Modified from Oyenihi et al. (2015))

(NF-kB). These mechanisms lead to β-cell death and dysfunction that eventually reduces insulin secretion (Oyenihi et al. 2015).

Meanwhile, the strong oxidizing agent H_2O_2 is produced following the reaction of superoxide dismutase (SOD)-catalyzed dismutation of O_2^-. These mechanisms may produce severe consequences of hydroxyl radical production, such as impaired vasodilation, leading to endothelial damage and tissue hypoxia (Vincent et al. 2004). The activity of free radicals, including O_2^- and H_2O_2, can be demonstrated through the levels of malondialdehyde (MDA) representing lipid peroxidation as a result of ROS activity attacking the lipid in plasma, endoplasmic reticulum, and mitochondria (Vincent et al. 2004; Ayala et al. 2014).

Previous studies revealed the consistent increase of MDA levels in the serum (Eissa et al. 2017), spinal cord (Ismail et al. 2018a), brain (Adefegha et al. 2021), and kidney (Kapucu 2021) of DN rats demonstrated the aberrant oxidative damage in these organs or fluids. In neonate DN rats induced by STZ at 90 mg/kg, the decreased SOD and glutathione peroxidase (GSHPx) activities indicating the occurrence of oxidative-nitrosative stress in the sciatic nerve have been reported which could be associated with the marked fall in motor and sensory nerve conduction velocity (NCV), mechanical allodynia, and thermal hyperalgesia (Kandhare et al. 2012).

Adiponectin

Adiponectin is secreted by the adipocytes, and it is documented that adiponectin gene polymorphism and glypican production give significant contribution to the pathogenesis of DN. In normal conditions, this hormone ensures the homeostasis of blood glucose circulation, lipid oxidation, insulin sensitivity, atherosclerosis, and coronary heart disease through the regulation of specific biomarkers (Adki and Kulkarni 2020; Tu et al. 2020). It is reported that the increased levels of adiponectin are positively linked with the occurrence of painful DN (Cha et al. 2018). Eissa et al. (2017) showed a negative correlation between the adiponectin level and glucose concentration. This study postulates that hyperglycemia does not lead to decreased adiponectin release. In a rat model of type II DN, a marked decrease of serum adiponectin was detected in the DN group compared to the control group by 63.01%. The study also revealed the reduced serum peroxisome proliferator activated receptor-gamma (PPAR-γ) levels, imbalanced oxidant-antioxidant levels (i.e., 4.78-fold increase of MDA with reduction of GSH level by 31.3%) in sub-abdominal adipose tissue and increased pro-inflammatory serum tumor necrosis factor-α (TNF-α) levels (Eissa et al. 2017). This study agreed that adiponectin plays significant action as antioxidant, anti-inflammatory and anti-atherogenic agents in physiological conditions as it responsibly regulates the production of the associated markers. The development of DN has lowered the secretion of adiponectin from adipose tissues, thereby pathologically altering the levels of the associated biomarkers. This postulation is also supported by Tu et al. (2020) identifying the gene expression profiling of certain biomarkers involved in the early stages of peripheral neuropathy in the sciatic nerve of the diabetic rat. The microarray analysis that was performed in the 6th week post-diabetic induction identified several important genes including *Adipoq* (i.e., adiponectin) which was downregulated by 4.348-fold in the

peripheral sciatic nerve. The significant decrease in paw withdrawal threshold (PWT) indicating tactile allodynia and reduced motor NCV have strengthened the connection of these alterations to the impaired role of adiponectin in regulating inflammatory, atherogenic, and diabetic mechanisms due to its low production during the early phases of DN.

Immune Cells

Glial Cells in Peripheral and Central Nervous Systems

A growing body of evidence revealed that the non-neuronal cells such as microglia and astrocytes become activated under hyperglycemic environment during the pathophysiology of DN. Previous literatures have exhibited strong glial-neuron crosstalk through the release of several inflammatory mediators leading to pathological pain, including allodynia and hyperalgesia in DN rat models. In a study by Barragán-Iglesias et al. (2018), the DN neonate Wistar rat model demonstrated increased glial fibrillary acidic protein (GFAP) and OX-42 immunoreactivity (remarks astrocyte and microglia expressions, respectively) with the marked increase of hypertrophied and amoeboid-like structure of microglia in the spinal cord as well as increased satellite glial cells (SGCs) in the DRG neuron at 16 weeks following the STZ injection (70 mg/kg). The enhanced expression of these glial cells peripherally and centrally has been found to be closely associated with the development of tactile allodynia in this neonate rat model, therefore, justifying their significant contributions during the pathogenesis of DN. In another study, Ismail et al. (2020) also revealed the enhanced expression of OX-42 positive cells in the spinal cord of painful DN rat model at the early stages that possibly explained the enhanced formalin-induced pain behavior responses in this rat model. The study also demonstrated an increase in spinal brain-derived neurotrophic factor (BDNF) and downstream regulatory element antagonist modulator (DREAM) immunoreactivities that are probably linked to the increase in microglia-neuron crosstalk since DREAM is transcribed in the neuron following the increased calcium ion (Ca^{2+}) influx from the activated *N*-methyl-D-aspartate receptors (NMDARs). The attenuation of microglia activation together with BDNF and DREAM signaling proteins by minocycline seems to reverse the allodynic and hyperalgesic state in painful DN rats (Ismail et al. 2019). The results were found to be contradictory in the painless DN rat as the expressions of microglia and the signaling proteins were reduced, therefore, remarking the significant roles of microglia-neuron crosstalk in the pathogenesis of DN. Meanwhile, Bishnoi et al. (2011) revealed a similar upregulation of OX42 staining expression in laminae I and II of the spinal dorsal horn. However, the increased microglia expression is suggested to be induced by STZ injection rather than as a result of hyperglycemia.

In addition, Lu et al. (2017) developed the diabetic rat model to investigate the involvement of α_2-adrenergic receptor in DN. Mechanical and thermal withdrawal latency tests (MWT and TWL) have been conducted to assess mechanical and thermal hyperalgesia. The spinal cord was collected to determine the expressions

of microglia, astrocytes, inflammatory cytokines (TNF-α and IL-1), glutamate levels, and neuronal apoptosis. It has been shown that the DN rats have a reduced pain threshold for mechanical and thermal stimuli, enhanced activation of microglia but not astrocytes, increased pro-inflammatory markers, enhanced apoptosis as proven by the increased cleaved-caspase-3 expression and increased glutamate levels. There was no change of astrocyte activation reported in another study by Nardin et al. (2016) investigating the astrocytic changes in the rat hippocampus. However, the specific astroglial abnormalities were detected in this brain region as the S100B content, a protein secreted mainly by the astrocytes, decreased in the hippocampus but increased the S100B secretion in blood serum. The GluN1 subunit of the NMDA receptor for glutamate was downregulated in the hippocampus of the diabetic rat. This study proposes that the astroglial abnormal changes along with the decreased GluN1 contents could be linked to the failure of glutamatergic crosstalk in this rat model following the advanced glycation end product receptor (RAGE)-induced inflammation. Apart from that, Zhou et al. (2018) investigated the expression of $P2Y_{13}$ receptor, Iba-1 (representing activated microglia), interleukin-1β (IL-1β), interleukin-6 (IL-6), and NR2B of NMDARs (NR2B) expression in the spinal cord of DN rats at the early stages. The administration of $P2Y_{13}$ antagonist MRS221 has downregulated these protein expressions and thereby alleviating the mechanical allodynia. The authors believed that the activation of the P2Y receptor expressed on microglia merely activate these glial cells through the action of IL-6 on the Janus kinase-2/signal transducer and activator of transcription-3 (JAK2/STAT3) signaling pathway leading to further microglia activation. These effects may also stimulate the phosphorylation of NR2B, a subunit of NMDARs leading to its increased activation and central sensitization, thereby contributing to allodynia and hyperalgesia. The postulated glial-neuron communications based on the previous animal studies are summarized in Fig. 2.

Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB)

Another biomarker that plays key roles in the pathogenesis of DN is NF-κB, a pro-inflammatory mediator that is activated via canonical or non-canonical NF-κB pathways. This inflammatory protein commonly participates in stress response, adaptive immunity, lymphoid organogenesis, and B-cell development (Liu et al. 2017; Adki and Kulkarni 2020). The upregulation of NF-κB gene expression has been detected in the sciatic nerve (Yousefzadeh et al. 2015; Sun et al. 2019) and aorta (Li et al. 2016) of the DN rat. The pathophysiology leading to the NF-κB upregulation and its effects is simplified in Fig. 3. Following the inflammation is cellular damage or hyperglycemic state. NF-κB is believed to be activated by the other pro-inflammatory cytokines like TNF-α and IL-1β as well as oxidative stress markers through the binding of these cytokines on toll-like receptors (TLRs) via a canonical (classic) pathway (Lawrence 2009; Yu et al. 2012). Consequently, the IκBα degradation is triggered by a multi-subunit IκB kinase (IKK) complex via its site-specific phosphorylation. The IκB is the inhibitor family of NF-κB comprising IκBα, IκBβ, IκBɣ, and B-cell lymphoma-3 (Bcl-3) that keep NF-κB inactive in cytoplasm during normal physiology (Lawrence 2009). Meanwhile, the IKK

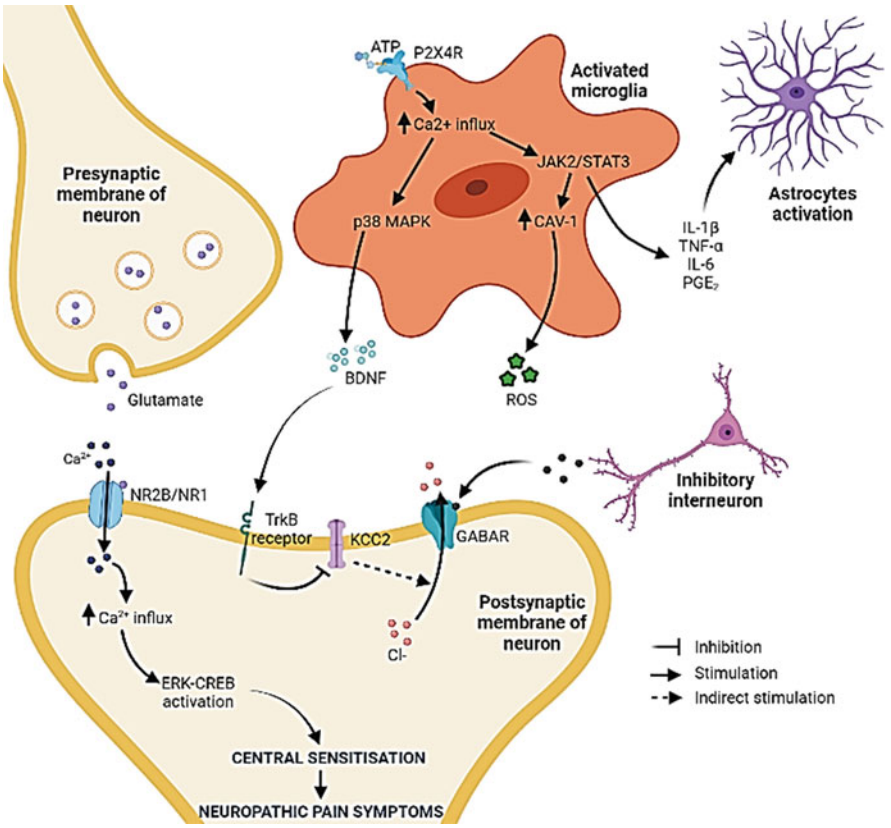

Fig. 2 Neuron-glia crosstalk during the pathophysiology of diabetic neuropathy (DN). Microglia is activated possibly via P2X4 receptor activation leading to the increased release of brain-derived neurotrophic factor (BDNF). Metabotropic P2Y receptor activation leads to the activation of Rho-associated kinase/p38 mitogen–activated protein kinase/nuclear factor kappa B (ROCK/p38 MAPK/NF-κB) signaling pathways which contribute to the release of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6. These inflammatory cytokines may also possibly contribute to the astrocytic modifications. The IL-6 may in turn prompt further microglial activation via Janus kinase-2/signal transducer and activator of transcription-3 (JAK2/STAT3) signaling pathway, possibly explains the hypertrophied morphology of activated microglia. The increased expression of caveolin-1 (CAV-1) leads to reactive oxygen species (ROS) production. Meanwhile, the increased release of BDNF may further bind to tyrosine kinase B (TrkB) receptor that downregulate the potassium-chloride transporter (KCC2) leading to the higher outflow of chloride ion (Cl^{-}) when γ-amino butyric acid (GABA) binds to its receptor in the spinal cord neurons. These mechanisms result in depolarization of the membrane potential and disinhibition. Moreover, temporal summation of postsynaptic depolarizations may lead to the removal of magnesium ion (Mg^{2+}) block from N-methyl-D-aspartate (NMDA) receptor (possibly NR2B) leading to the increased influx of Ca^{2+} into the post-synaptic neurons. In turn, these processes further activate protein kinase A, phosphorylate extracellular signal-regulated kinases/phosphorylated cAMP response element binding protein (ERK/pCREB) signaling pathways leading to transcriptional changes in the neuron. These orchestrated central modifications may lead central sensitization that probably explains the development of allodynia and hyperalgesia in DN

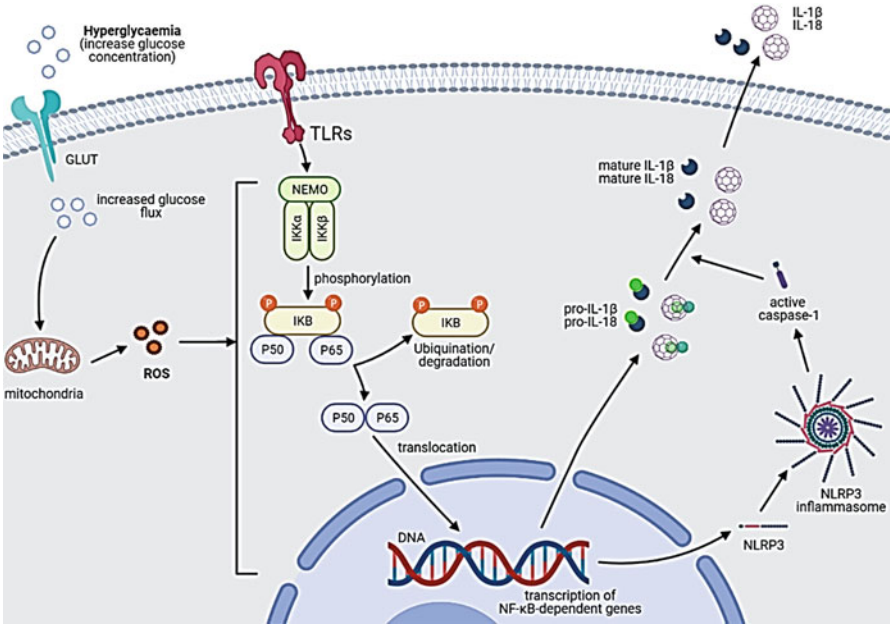

Fig. 3 The actions of NF-κB in causing overproduction of inflammatory markers. Hyperglycemic condition during diabetes leads to the production of ROS that induces the increased production of NF-κB in the affected cells. The binding of certain inflammatory biomarkers (TNF-α and IL-1β) to Toll-like receptors (TLRs) leads to the activation of NF-κB signaling through canonical (classic) pathway. The IκB kinase (IKK) activation results in the degradation of IκBα. The NF-κB members (p50/ReIA) translocate into the nucleus to induce NF-κB-dependent genes transcription including pro-IL-1β, pro-IL-18, and NLRP3. Upon the maturation by activated caspase-1, these inflammatory markers are secreted out to enhance the inflammatory insults. Adapted from Liu et al. (2017). ROS = reactive oxygen species, IL-1β = interleukin-1β, IL-18 = interleukin-18, NLRP3 = Nod-like receptor protein 3, NF-κB = nuclear factor kappa B cell

complex comprises two catalytically activated kinases, IKKα (IKK1) and IKKβ (IKK2), together with a regulatory scaffolding protein NF-κB essential modifier (NEMO) that keeps the NF-κB inactive in the cytoplasm (Suryavanshi and Kulkarni 2017). Following the activation, the IKK phosphorylates IκBα at two N-terminal serines that trigger the ubiquitin-dependent IκBα degradation in proteasome. In turn, this mechanism results in the rapid and transient nuclear translocation of canonical NF-κB members (i.e., p50/ReIA and p50/c-Rel dimers) (Lawrence 2009; Liu et al. 2017). In the nucleus, the activated NF-κB stimulates the transcription of NF-κB-dependent genes like Nod-like receptor protein 3 (NLRP3), pro-IL-1β, and pro-IL-18. The production of NLRP3 inflammasome, as evidently increased in the sciatic nerve of STZ-induced DN rat (Sun et al. 2019), may induce the activation of caspase-1 that further catalyzes the maturation of IL-1β ad IL-18 (Liu et al. 2017). In the occurrence of hyperglycemia, NF-κB is highly produced following TNF-α and IL-1β activations that subsequently trigger the secretion of other pro-inflammatory and immune mediators, including T cells and innate immunity cell. These effects

terminally cause endothelial cell damage (Suryavanshi and Kulkarni 2017). Aside from that, the co-localization of NF-κB p65 with NADPH oxygenase-2 (NOX2) and NADPH oxygenase-4 (NOX4) in the aorta of DN rats revealed the potential role of NF-κB in triggering the NOX activation and ROS overproduction following prolonged hyperglycemia (Li et al. 2016). Moreover, the transcription of NF-κB may also lead to enhanced production of heme oxygenase-1 (HO-1), a stress protein in the astrocyte culture of the rat's brain (Yang et al. 2017).

Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α)

The IL-1β and TNF-α are crucially known for not only mediating inflammation but also promoting cell apoptosis and death. Several cells are responsible to release IL-1β, TNF-α, and other inflammatory markers upon activation such as microglia and DRG neurons (Ismail et al. 2018b; Hemati et al. 2021). A number of studies have previously reported the significant roles of these inflammatory mediators in the pathogenesis of DN as they initiate and maintain the inflammation-induced organ failure in DM. Evidently, the IL-1β production is associated with the NF-κB activation following prolonged hyperglycemia. In the DN rat model, the marked increase of IL-1β, TNF-α, and/or other pro-inflammatory cytokine levels have been reported in the spinal cord (Bishnoi et al. 2011; Ismail et al. 2020), peripheral DRG neuron (Hemati et al. 2021), blood serum (Eissa et al. 2017), and sciatic nerve (Kandhare et al. 2012; Sun et al. 2019).

In a study mimicking insulin-dependent DM in a neonate rat model, Kandhare et al. (2012) measured the pro-inflammatory and oxidative stress markers in the sciatic nerve and their association to several tests evaluating mechanical, tactile, and thermal hyperalgesia as well as motor and sensory NCV. The findings exhibited a marked elevation of both IL-1β and TNF-α, reduced SOD and GSH activities and increased lipid peroxidation at 14 weeks post-diabetic induction. The significant reduction in mechanical, thermal, and tactile thresholds as well as sensory and motor NCV in DN neonate rats are possibly associated with the increased IL-1β and TNF-α levels and imbalanced oxidant-antioxidant activities. These effects therefore explain their contribution to the development of allodynia and hyperalgesia. It is possible that the increased levels of these pro-inflammatory cytokines are not caused by the STZ injection, but due to the hyperglycemic environment (Quan et al. 2011). Hemati et al. (2021) suggested that the increased pro-inflammatory cytokine levels in the DRG neurons of DN rat are through the increased activation of NMDAR, especially NR2B. Other than that, the significant elevated levels of these TNF-α and IL-1β were also observed in the spinal cord of DN adult rats (Bishnoi et al. 2011) and the painful variant of DN rats (Ismail et al. 2018a) at the early stages. It is believed that the increase of pro-inflammatory markers is contributed by the microglial activation or the elevated expression of transient receptor potential vanilloid-1 receptor (TRPV1) rather than caused by the STZ-induced hyperglycemic environment (Bishnoi et al. 2011).

Monocyte Chemoattractant Protein-1 (MCP-1)

The MCP-1 or also known as C-C motif ligand 2 (CCL2)/monocyte chemotactic and activation factor is a monomeric polypeptide that may amplify the excitatory

synaptic transmission during the development of DN. There are limited numbers of studies that looked at the role of MCP-1 in the pathogenesis of DN in the rat model. In an in vitro study applying rat primary microglia cell culture performed by Quan et al. (2011), the high glucose treatment enhanced NF-κB activation that consequently upregulates the TNF-α and MCP-1 mRNA expressions. The increased release of MCP-1 is believed to be closely associated with the increased microglia activation following prolonged hyperglycemia resulting in the excessive formation of ROS. In turn, the NF-κB signaling pathway is activated due to the actions of free radicals leading to the increased transcription of MCP-1 and other pro-inflammatory cytokines. The inflammatory markers such as TNF-α, interferon-γ, IL-1β, and platelet-derived growth factors are also believed to stimulate macrophages, monocytes, and dendritic cells to secrete MCP-1 that add up to its excessive level (Adki and Kulkarni 2020). Meanwhile, there is another study investigating the astrocytic-derived MCP-1 in the DN rat model, but in paclitaxel-induced peripheral neuropathy rats where the MCP-1 expression is enhanced in the small nociceptive DRG neurons and spinal astrocytes (Zhang et al. 2013). In the spinal cord, the upregulated expression of MCP-1 is postulated to occur from the activation of c-Jun-N-terminal kinase (JNK) by TNF-α via its binding to TNF receptor-1 (Adki and Kulkarni 2020).

Cell Receptors

Transient Receptor Potential Vanilloid-1 (TRPV1)

The TRPV1 is a transducer polymodal protein that is predominantly found in the nociceptive neurons of the peripheral nervous system and certain tissues of the central nervous system. Previous literatures revealed the significant association of TRPV1 activation with the noxious thermal and inflammatory stimulation following the activation of capsaicin, phosphatidylinositol, and tyrosine kinase in the peripheral and nociceptive neurons (Smith et al. 2002; Adki and Kulkarni 2020). Bishnoi et al. (2011) discovered that the TRPV1 expression was enhanced at the central terminals of the spinal cord dorsal horn in which the DN rats also developed thermal and mechanical hyperalgesia following 1 week and 5 weeks after STZ injection, respectively. The authors claimed that these aberrant TRPV1 expressions are contributed possibly by the direct effects of STZ rather than the hyperglycemic state. Meanwhile, similar enhanced immunoreactivity of TRPV1 was also reported by Hong et al. (2008) in the large DRG neurons of DN rats. The authors suggested that the repeated TRPV1 activation leads to overload of Ca^{2+} influx that results in the stimulation of pathways linked with caspase- and calpain-dependent oxidative stress. These mechanisms eventually contribute to apoptotic cell injury and neuronal stress in DRG neurons during the early phases of diabetic sensory neuropathy. In another study contradicting Bishnoi et al. (2011), Zhang et al. (2019) reported that the varying degrees of TRPV1 expression reduction in the STZ-induced DN rat compared to the non-diabetic rat possibly explained the reduced NCV in the peripheral sciatic nerve of this rat model. The existing discrepancy is possibly due to the morphological and electrophysiological changes that occurred due to the different

stages of DN in each study as Bishnoi et al. (2011) developed the early stages of DN rats (5 weeks following STZ injection) while Zhang et al. (2019) developed more severe stages of DN (8 weeks following STZ injection).

N-Methyl-D-Aspartate Receptors (NMDARs)

The NMDARs are the heteromeric protein complexes comprising three subunits: NR1, NR2, and NR3. Each subunit comprises 1–7 subunit types that play specific physiological roles in regulating pain, memory, and synaptic development. It has been reported that NMDARs activity is exaggerated at both the peripheral and central levels during the pathogenesis of DN (Bai et al. 2014; Chen et al. 2016; Li et al. 2019; Hemati et al. 2021).

Previous studies have linked the activation of NMDAR to the development of hyperalgesia in several neuropathic pain conditions including DN. Bai et al. (2014) have reported that the NR2B mRNA and protein expressions were upregulated in the spinal cord of STZ-induced DN rat model which was associated with the development of hyperalgesia as indicated by the reduced PWT. It was postulated that the enhanced activity of NR2B is contributed by the reduced activation of ɣ-amino butyric acid B ($GABA_B$) receptor activation. The increased NR2B activity leads to the increased phosphorylation of CREB that further explains the development of hyperalgesia. It is also agreed by Liu et al. (2014) that these mechanisms are contributed by the downregulation of $GABA_B$ receptors as the injection of baclofen, a $GABA_B$ receptor antagonist, has normalized the NMDAR expression level, and therefore improving the hyperalgesic condition in this rat model (Bai et al. 2014). In another study, Hemati et al. (2021) aimed to investigate the possible inhibitory pathway of atorvastatin to attenuate the progression of DN in diabetic rats. Hot-plate and formalin tests were carried out to evaluate thermal and chemical hyperalgesia respectively, with the measurements of several pro-inflammatory cytokines and oxidative marker levels in the DRG neuron. The results revealed that the injection of NMDA agonist exerted hyperalgesia in the atorvastatin-treated DN rat. The levels of GluN2B and GluN1 representing NR2B and NR1 mRNA expressions together with pro-inflammatory markers were markedly elevated in the DRG neuron of the DN rat although no changes were reported in the oxidative stress activities. This study concluded that atorvastatin attenuated the progression of DN symptoms by inhibiting NMDAR activation and lowering the pro-inflammatory cytokine levels. Furthermore, the amplified activation of NR2B did not only occur at the peripheral nerves as Li et al. (2019) reported that the upregulated phosphorylation of NR2B expression remarked the augmented NR2B activation in the spinal cord of DN rats and neuronal cell culture, which is possibly activated via the microglial JAK2/STAT3 signaling pathway. A similar increase in NR2B activation was also reported by Chen et al. (2020) in the spinal cord of type II DN rats which further promote the aberrant production of ROS via the activation of caveolin-1 (CAV-1).

Gamma-Amino Butyric Acid (GABA) Receptors

The GABA receptor is a metabotropic transmembrane receptor that is linked through G-proteins to potassium channels (Liu et al. 2014). This receptor functions as

autoreceptor for feedback mechanism of neurotransmitter GABA release and as a heteroreceptor that controls synaptic glutamate and glycine release to the spinal cord dorsal horn (Liao et al. 2017). The spinal GABAergic inhibitory impairment is known to have critical contributions to the pathogenesis of neuropathic pain.

In neuropathic state, the activation of $GABA_A$ receptors is impaired since the abnormal changes in the extracellular GABA levels occurred. The study conducted by Afrazi and Esmaeili-Mahani (2014) demonstrated that the mRNA expression of $GABA_A$ receptor was downregulated in the spinal cord of the painful DN rats, which was also associated with the lower threshold of the tail-flick test and motor coordination indicating the occurrence of thermal hyperalgesia and motor deficits. The treatment by allopregnanolone has ameliorated these abnormal changes and improved the neuropathic condition. In another study, Lee Kubli et al. (2021) investigated the rate of depression (RDD) in the spinal H-reflex of DN rats that was impaired following 8 weeks of diabetic induction. Further investigations revealed the downregulation of KCC2 expression in the spinal cord as early as 4 weeks post-diabetic induction which suggests the progressive impairment of $GABA_B$ receptor-mediated inhibition contributing to spinal disinhibition. The authors postulated that the GABAergic-inhibition, either from $GABA_A$ or $GABA_B$ receptor-mediated inhibition, possibly contributes to the normal RDD whilst the progression from normal to impaired RDD during DN could be due to the impairment of either one of these two receptors. In addition, Liao et al. (2017) investigated the possible underlying mechanism of surgical treatment by chronic nerve compression in painful DN rats in regard to its effects on certain behavior responses and $GABA_B$ receptor expressions. The results showed that the demyelination of primary afferent central terminal in the sciatic nerve occurred with the reduced expression of $GABA_B$ receptors detected in those areas in the painful DN rat with mechanical allodynia. The authors concluded that the myelinated primary A-afferent fibers are mostly impaired rather than the unmyelinated C-fibers following chronic nerve compression in the painful DN rat. Additionally, the downregulated $GABA_B$ receptor in the spinal cord could be one of the factors contributing to the occurrence of mechanical allodynia in this rat model.

Enzyme-Related Markers

Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NOX)

The NOXs are multicomponent enzymes that primarily facilitate the electron transfer from cytosolic NADPH to molecular oxygen. These enzymes are available in the vascular endothelial cells, microglia, neurons, and astrocytes of the brain. Evidence have revealed the role of NOXs in mediating the development of neuropathic pain through the overproduction of oxidative species, mainly O_2^- and H_2O_2 in the phagocytic cells (Li et al. 2016; Ji et al. 2017; Adki and Kulkarni 2020). Among the NOXs family (i.e., NOX1-NOX5, DUOX1-DUOX2) (Li et al. 2016), NOX4 is mostly linked to diabetic complications (Ji et al. 2017) as long-term hyperglycemia causes NOX4 upregulation through the tyrosine kinase and mitogen-activated

protein kinase (MAPK) pathway (Adki and Kulkarni 2020; Laddha and Kulkarni 2020). Furthermore, the overproduction of ROS is also believed to trigger the NOX4 activation that acts via angiotensin II to develop peroxynitrite (strong oxidant) in endothelial nitric oxide synthase uncoupling in nerve tissues (Laddha and Kulkarni 2020). Consequently, the NOX4 overexpression is linked to aldose reductase, AGEs, NF-κB, IL-6, IL-18, and endothelial growth factor activation, collagen deposition, and hyperlipidemia (Laddha and Kulkarni 2020). Ji et al. (2017) have investigated the role of NOX in the bupivacaine-induced sciatic nerve injury in type II DN rats by administering diphenyleneiodonium chloride (NOX inhibitor). Hindpaw allodynia responses, PWT and NCV were performed, and sciatic nerve was collected for measuring NOX4 and other oxidative stress levels. It was found that the NCV was markedly reduced, and PWT was prominently higher with development of allodynia and abnormal sciatic nerve morphology with the presence of axonal atrophy, severe segmental demyelination, and nerve fiber loss in the DN rat. It is postulated that these abnormal functional and morphological changes are contributed by the enhanced levels of lipid peroxide and hydroperoxide together with the upregulated NOX2, NOX4, and caspase-3 expressions in the sciatic nerve leading to ROS overproduction and cell apoptosis. Similar results were also obtained by Li et al. (2016) in which they discovered that the mRNA expression of NOX2 and NOX4 was significantly elevated in the aorta of the STZ-induced DN rat. The immunohistochemistry analysis also revealed the significant increase of these biomarkers together with NF-κB, co-localized in the smooth muscle of the DN rat's aorta. In an in vitro study applying Schwann cells of newborn Wistar rat, Yu et al. (2019) discovered that the high glucose condition has upregulated the NOX4 mRNA and protein expressions, thereby reducing the activity of Schwann cells, elevating the intracellular ROS level and inducing cell apoptosis through the increased caspase-3 mRNA and protein expressions.

Heme Oxygenase-1 (HO-1)

The HO-1 is an inducible isoenzyme that is lowly and limitedly expressed in the scattered neurons and neuroglia during normal homeostasis (Chen et al. 2016; Yang et al. 2017). Several stressful and neuropathological conditions such as Alzheimer's, Parkinson's, and diabetes could induce the overproduction of HO-1. In fact, HO-1 and heme degradation play pleiotropic effects in minimizing injuries caused by many disease complications via its antioxidant, angiogenesis, and anti-inflammatory actions (Chen et al. 2016). This enzyme degrades heme into ferrous ion, biliverdin that acts as strong antioxidants, and carbon monoxide which halts multiple biological functions including oxidative stress production and inflammation (Chen et al. 2016).

There are limited studies that investigated the role of HO-1 in the DN rat model, but it has been portrayed that HO-1 plays a protective role during the pathogenesis of DN as demonstrated in in vivo and in vitro studies. In the rat brain astrocytes, Yang et al. (2017) reported that HO-1 mRNA and protein expressions are amplified by the high levels of glucose in a dose-dependent manner as the higher glucose environment results in higher expressions of HO-1. It is possible that the

hyperglycemic-induced HO-1 expression is activated by a ROS-dependent signaling mechanism as the NOX- and mitochondrion-dependent ROS development leads to the activation of ERK1/2. These effects further activate downstream transcriptional factors, NF-κB. Meanwhile, excessive ROS also stimulates the JNK pathway leading to the transcription of c-fos/activator protein-1 (AP-1). Consequently, both of these activated signaling pathways simultaneously switch on the transcription of HO-1 gene that eventually results in neuronal apoptosis. Meanwhile, Chen et al. (2016) developed a diabetic wound rat model treated with hemin (HO-1 inductor agent) for 21 days. The HO-1 significantly speeds up the wound closure rates after 5 days of wounding possibly by promoting the re-epithelialization in the diabetic rat. The application of hemin boosted the endogenous HO-1 protein expression possibly by increasing the activity of antioxidant SOD and minimizing the occurrence of lipid peroxidation in the wound tissue of the diabetic rat. Besides that, the application of hemin also stimulated angiogenesis and lowered the angiogenesis-inhibiting cytokines like vascular endothelial growth factor (VEGF) and intracellular adhesion molecule-1 (ICAM-1) in the small vessels surrounding wounded tissue of the diabetic rat. The aforementioned studies demonstrated the protective role of HO-1 during the pathophysiology of DN in order to alleviate the DN complications.

Other Protein

Caveolin-1 (CAV-1)

The CAV-1 is a major constituent of caveolae that controls the neuronal plasticity and receptor transport to regulate NR2B of NMDARs activation leading to pathological modifications and central sensitization (Li et al. 2019; Adki and Kulkarni 2020). Several studies have reported the significant contribution of CAV-1 in the pathogenesis of DN. Li et al. (2019) conducted a combined in vivo and in vitro study to investigate the role of JAK2/STAT3-CAV-1-NR2B signaling pathways in the spinal dorsal horn of the painful DN rat model. There were higher expressions of total CAV-1, phosphorylated JAK2, STAT3, and NR2B in the spinal cord that were highly associated with the enhanced mechanical and thermal hypersensitivity in this rat. The expression of CAV-1 and phosphorylated NR2B were also upregulated in the neuronal cell culture, possibly contributed by the activation of phosphorylated STAT3 in the microglia cell line following the high-glucose state. The study concluded that the neuropathic pain condition is contributed by the activated CAV-1-NR2B pathway as influenced by the activation of microglial JAK2/STAT3. Similar results were also obtained by Chen et al. (2020) but suggesting different pathways that contribute to CAV-1 upregulated expression. In their study, phosphorylated CAV-1 continuously increased and is activated in the spinal cord, possibly linked to the lowered mechanical and thermal threshold in the rat. It is suggested that the enhanced spinal CAV-1 expression is modulated by the recombinant Human Ras/Related C1/nicotinamide adenosine diphosphate oxidase-2/NR2B gene (Rac1/NOX2-ROS-NR2B) pathway leading to central sensitization and development of DN.

Conclusion

Several biomarkers have been identified to play predominant roles in the pathogenesis of DN. These biomarkers could be applied as diagnostic tools for early detection and prediction of disease progression. Certain pathways involving these biomarkers can be targeted to develop specific drugs to combat DN. The establishment of the DN rat model benefits the researchers to understand the pathogenesis of the disease progression and therefore modulate certain pathways to combat or slow down disease progression.

Applications to Prognosis, Other Diseases or Conditions

Understanding the underlying pathogenesis of DN in a rat model may provide rationales to target specific pathways to treat when taking into account the duration and stages of the disease. Although it could not completely mimic the specific pathomechanisms as in humans at the molecular level, the pathological modifications that occurred during the development of DN in the rat model sufficiently provide a real scenario that possibly occurs in humans. Evidently, modern and alternative medications currently used to alleviate neuropathic symptoms in DN patients such as pregabalin, gabapentin, amitryptillin, desipramine, and capsaicin have initially undergone preclinical phases to identify their effectiveness before being selected for several clinical phases. By having the DN rat model, new therapeutics could be developed targeting specific pathways in order to improve and alleviate the neuropathic symptoms in human. The positive outcomes by developing the DN rat model may therefore improve the quality of health care through the productions of several quality medications and indirectly reduce the socioeconomic burden of a country.

Mini-dictionary of Terms

Atherogenic Process that promotes the fatty deposition in the blood arteries
Hyperalgesia Heightened pain sensation
Allodynia Pain resulted from the stimulus that does not usually cause pain
Nociceptive Process related to pain perception
Pathophysiology Abnormal physiological process related to a disease or injury
Neuropathy Impairment of a nerve leading to tingling or numbness sensations
Sensitization Increased responsiveness to pain

Key Facts

Key Facts of Diabetic Neuropathy

- The molecular modifications either at the central or peripheral nervous systems during the pathogenesis of DN is believed to originate from chronic hyperglycemia. Controlling the glucose level by changing the lifestyle and taking pain medications seems to be the most effective way to control the disease progression.

- Painless DN could be the late stage of DN since most of the small and large nerves are impaired leading to loss of sensation. The effects could eventually lead to foot ulcers and limb amputations.

Key Facts of Rat Model of Diabetic Neuropathy

- Several rodent models have been developed to mimic DN for decades such as genetically modified C57BL/Ks (db/db) mice, spontaneously diabetic WBN/Kob rats, L-fucose induced neuropathic rat. However, most of these models were not validated for antidiabetic or anti-neuropathic medications.
- Nerve damage is the common symptom in DN. The rat models of DN mostly revealed the reduced motor and/or sensory nerve conduction velocity indicating the structural and functional impairment of nerves.
- The STZ-induced DN rat model has been widely used in research to mimic DN complications as in humans due to its potent destructive ability on pancreatic β-cells. The lower dose of STZ combined with high-fat diet may result in insulin resistance as in type II DM in a rat model.

Summary Points

- DN can be developed from chronic hyperglycemia either due to the pancreatic β-cell destruction in type I DM or insulin resistance from type II DM.
- The metabolic modifications during DN could be contributed by the orchestrated effects of inflammatory, nociceptive, oxidative stress, and enzymatic biomarkers leading to multiple changes at the central and peripheral nervous systems.
- The increase in neuron-glia crosstalk has been demonstrated in several neuropathic pain states including DN. These communications lead to the development of central sensitization, therefore, explaining the development of allodynia and hyperalgesia.
- Certain biomarkers are downregulated during normal physiology, but highly expressed during the disease state. These biomarkers such as HO-1 are believed to be the endogenous protective mechanisms to bring the body regulations back to normal.
- Investigating molecular mechanisms to understand the pathogenesis of DN in diabetic rat models gives insights to develop several therapeutic drugs and alternative medicines alleviating neuropathic symptoms.

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Biomarkers in Experimental Diabetes: Studies with *Syzygium Cumini* (L.) and Links with the Sulfonylurea 1 Receptor

54

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Contents

Introduction 1114
Pathophysiology of Diabetes Mellitus 1114
Clinically Used Biomarkers in Diabetes Mellitus 1116
Animal Models for Experimental Diabetes 1116
Experimental Models for Type 1 DM 1116
Experimental Models for Type 2 DM 1119
Experiments with *Syzygium Cumini* 1119
Fasting Blood Glucose (FBG) 1119
Serum Insulin and C Peptide Levels 1121
Glycosylated Hemoglobin (HbA1C) 1121
Homeostatic Model of Assessment (HOMA) 1122
Liver Glycogen 1122
Lipid Profile 1123
Adiponectin 1124
Glucagon-Like Peptide 1 (GLP 1) 1124
Markers of Oxidative Stress 1124
Inflammatory Biomarkers 1125
Histopathology of Islets of Pancreas 1126

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PPARs (Peroxisome Proliferator-Activated Receptors) 1126
Hexokinase 1128
Biomarkers for Evaluating Molecular Mechanisms 1129
Molecular Docking Study of *Syzygium Cumini* extract Components on Sulfonylurea Receptor (SUR1) 1129
Applications to Prognosis 1130
Mini Dictionary of Terms 1130
Key Facts: Biomarkers in Experimental Diabetes: Studies with *Syzygium Cumini* (L.) and Links with the Sulfonylurea 1 Receptor 1133
Summary 1134
References 1134

Abstract

Diabetes mellitus (DM) is showing a global rise in its incidence. Pancreatic beta cell dysfunction, insulin resistance, and inflammation contribute to hyperglycemia and other metabolic derangements. Uncontrolled DM leads to micro- and macrovascular complications. Experimental animal models that closely simulate the human disease with identifiable biomarkers have been standardized for developing newer therapies for DM. While the conventional biomarkers such as fasting/postprandial blood glucose and HbA1C measure the extent of hyperglycemia, there are newer biomarkers for measuring the beta cell dysfunction, insulin resistance, oxidative stress, and inflammation. The expression (protein and gene) of certain important enzymes, receptors, and intracellular signaling molecules such as PPAR γ, GLUT 4, GLUT 2, etc., are also being considered. *Syzygium cumini* (SC) is a medicinal plant that has undergone extensive research over 100 years in experimental models of DM and is still being explored. This chapter describes various biomarkers in experimental models of DM with illustrations from experiments on SC.

Keywords

Syzygium Cumini (L.) · Sulfonylurea 1 receptor · High fat diet-streptozotocin · Alloxan · Fasting Blood Glucose · HbA1C · Plasma Insulin · Liver Glycogen · HOMA-IR · HOMA- β · Lipid profile · Oxidative Stress Markers · TNF- α · PPAR- γ · GLUT-2 and 4 · Islet cell regeneration

Abbreviations

8-OHdG	8-OH deoxyguanosine
AGE	Advanced glycated End Product
AI	Artificial Intelligence
AOPP	Advanced Oxidative Protein Products
BB	Bio Breeding
CAD	Coronary Artery Disease
CD4	Cluster of Differentiation 4
CD8 T cells	Cluster of Differentiation 8 T cells

CD8	Cluster of Differentiation 8
CRP	C-Reactive Protein
CVA	Cerebro Vascular Accident
DM	Diabetes Mellitus
DPP-4	Dipeptidyl Peptidase-4
EJ	*Eugenia Jambolana*
ELISA	Enzyme-Linked Immunosorbent Assay
FBG	Fasting Blood Glucose
GLP1	Glucagon-Like Peptide 1
GLUT-2	Glucose Transporter type 2
GLUT-4	Glucose Transporter type 4
GPLD1	Glycosylphosphatidylinositol-Specific Phospholipase D1
HDL	High-Density Lipoprotein
HFD	High Fat Diet
HFHS	High carbohydrate, high fat diet
HOMA	Homeostasis Model of Assessment
IA-2	Insulinoma-Associated protein 2
ICAM-1	Intercellular Adhesion Molecule-1
IL-18	Interleukin-18
IL-1RA	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IR	Insulin resistance
LDL	Low density Lipoprotein
Lp(A)	Lipoprotein(A)
MDA	Malondialdehyde
miRNA	Micro Ribonucleic acid
NOD	Nonobese diabetic
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
PEDF	Pigment Epithelium-Derived Factor
PI3K	Phospho Inositide 3 Kinase
PKC	Protein Kinase C
PPARγ	Peroxisome proliferator- activated receptor gamma
PPBG	Postprandial blood glucose
ROS	Reactive Oxygen Species
SC	*Syzygium cumini*
SGLT2	Sodium-glucose transport protein 2
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
STZ	Streptozotocin
SUR1	Sulfonylurea receptor1
TBARS	Thiobarbituric acid-reactive substances
TGF-β	Transforming growth factor β
THBS1	Thrombospondin 1

TNF-α	Tumor Necrosis Factor α
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
α-Hb	α- Hemoglobin

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by increase in the blood glucose. Type 1 DM is due to an autoimmune destruction of beta cells, while over 90% of DM cases are type 2, characterized by deficient pancreatic insulin secretion and tissue insulin resistance (IR) (Paschou et al. 2018; Galicia-Garcia et al. 2020).

Experimental models are developed to study the pathogenesis and evaluate newer therapies for DM. Spontaneous and genetic models are mostly used for type 1DM, though it can also be induced with chemicals or viruses. However their relevance to the human disease has been questioned (Phillips et al. 2011). On the other hand, elaborate models for type 2 DM have evolved over a period of time mimicking the lifestyle and diet patterns that lead to human disease. More than 20 biomarkers are identified in humans and in experimental models for type 2 DM. With the development in technology, several proteomic and genomic biomarkers are being identified.

The emerging experimental pharmacological data on newer therapies for type 2 DM has shown that herbal extracts may be beneficial by acting at different targets due to multiple phytoconstituents. *Syzygium cumini* (*SC)*, also called *Eugenia Jambolana (EJ)*, is a medicinal plant native to the Asian subcontinent. It has shown insulin secretory, insulin sensitizing, antioxidant, and anti-inflammatory activity (Sharma et al. 2012; Sankhari et al. 2012). It is one of the plants that has undergone more than 100 years of research in experimental DM, and more than 100 case reports were published before the advent of insulin (Helmstädter 2008).

In this chapter, we first describe the current understanding of the pathophysiology of human diabetes and some of the clinically used biomarkers. We then discuss the experimental diabetic models used to evaluate the antidiabetic effects of *Syzygium cumini* emphasizing on the biomarkers that reflected the response to treatment.

Pathophysiology of Diabetes Mellitus

DM is multifactorial in origin and many organs are involved in its pathogenesis. In type 1 DM, there is an autoimmunity against β cells of the islets of pancreas. The process occurs in genetically susceptible individuals triggered by environmental factors such as viral infection, but positive for relevant autoantibodies. An increase in T lymphocytes, particularly CD4 and CD8, is observed in type 1 DM (Paschou et al. 2018).

Type 2 DM is multifactorial in its etiology. Both genetic predisposition and environmental factors play a role. Glucotoxicity and adipotoxicity resulting from

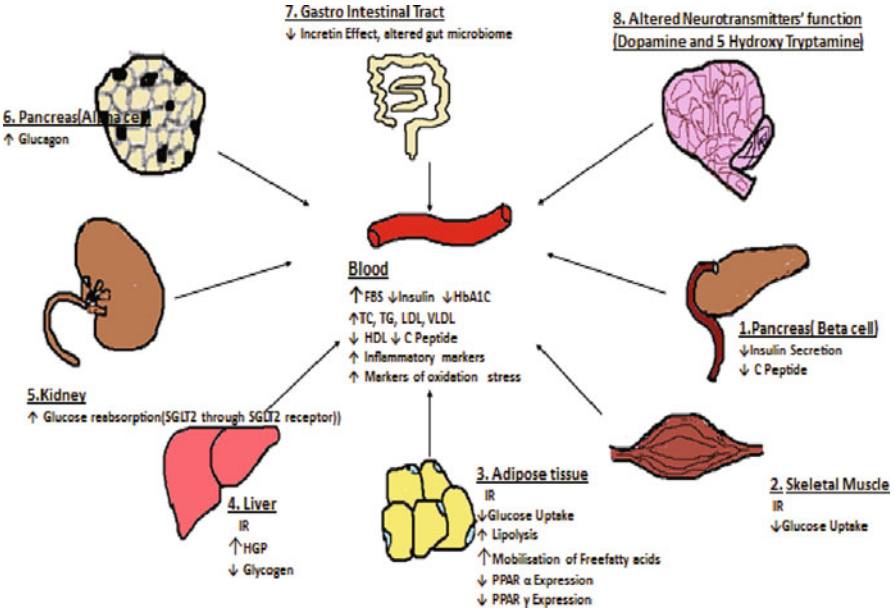

Fig. 1 Pathophysiological processes with biomarkers contributing to the metabolic derangements in type 2 DM. *IR* insulin resistance, *PPAR* peroxisome proliferator activated receptor, *HGP* hepatic glucose production, *SGLT* sodium glucose transporter, *FBG* fasting blood glucose, *TC* total cholesterol, *TG* triglycerides, *LDL* low density lipoprotein, *HDL* high density lipoprotein, *VLDL* very low density lipoprotein. (1) Decreased insulin secretion by the β cells due to autoimmune destruction and IR; (2) Decreased glucose uptake in the skeletal muscles due to IR (decreased GLUT 4 expression and altered insulin signaling pathways); (3) IR in adipose tissue leads to increased mobilization of free fatty acids, decreased expression of adiponectin and altered intracellular insulin signaling pathways. IR (decreased GLUT 4, decreased PPAR γ, and PPAR α expression); (4) Increased glucose hepatic production due to IR (decreased glycogen synthesis and increased neoglucogenesis); (5) Increased renal glucose reabsorption by SGLT2 and increased threshold for glucose secretion in the urine; (6) Increased glucagon secretion by α cells of pancreas; (7) Decreased incretin, altered gut microbiome; (8) Low dopamine, increased brain 5 hydroxytryptamine, resistance to the appetite, suppressive effects of insulin, leptin, GLP1, amylin, and peptide YY

deregulated diet and lifestyle are the precipitating factors in genetically susceptible individuals. Adipokine dysregulation, oxidative stress, abnormalities in gut microbiota, immune dysregulation, and inflammation have emerged as critical pathophysiological factors (Weyer et al. 1999).

Pancreatic β-cell dysfunction, insulin resistance, and chronic inflammation are the characteristic pathophysiological features of type 2 DM that lead to a state of hyperglycemia (DeFronzo 2010). Uncontrolled DM leads to micro- and microvascular complications such as neuropathy, nephropathy, and retinopathy, cardiovascular and cerebrovascular accidents. Metabolic molecular derangements that contribute to diabetic complication are identified to involve polyol pathway, stimulation of protein kinase C (PKC) isoforms, increased formation of advanced glycation end

products (AGEs) and increased expression of its receptor and overactivity of the hexosamine pathway(DeFronzo et al. 2015). Figure 1 below describes the pathophysiology based on the principles of ominous octet proposed and validated by DeFronzo et al. (2015). In addition, the authors also added vascular insulin resistance and inflammation that lead to the diabetic complications making it as "decadent decoplet" (DeFronzo et al. 2015).

Clinically Used Biomarkers in Diabetes Mellitus

Identifying biomarkers denoting the pathophysiological changes and responses to therapy play a significant role in detecting, predicting the progression of diabetes, and the development of complications (Dorcely et al. 2017).

Currently, in clinical practice, fasting blood glucose levels, 2-h blood glucose in a 75-g oral glucose tolerance (OGT), HbA1C have been the most preferred biomarkers recommended to diagnose and monitor the disease progression.

Several biomarkers representing the development of insulin resistance and the prediabetic state have been used to predict disease progression toward type 2 DM. Recently, novel biomarkers denoting the proinflammatory, metabolic, inflammatory, oxidative damage, genetic derangements, amylin accumulation, lipotoxicity, glucotoxicity, impaired incretin secretion, and decreased beta cell mass have been used. Figure 2 shows the biomarkers that have been studied and partially validated in DM (Dorcely). However they are not validated in terms of clinical response.

Animal Models for Experimental Diabetes

Experimental DM models have evolved over years of research on diabetes mellitus. Both genetic and nongenetic models have been developed based on the understanding of pathophysiology of DM. Streptozotocin (STZ) and alloxan-induced DM is common for both type 1 and type 2 DM. Animal models are broadly categorized into four groups – genetic, spontaneous, chemically induced, and virus induced. All the four are applicable to both types of diabetes.

Experimental Models for Type 1 DM

The main characteristic feature of type 1 DM is the destruction of the pancreatic beta cells, leading to lack of insulin production (Paschou et al. 2018). In animal models, this deficiency in insulin production is achieved by various mechanisms, ranging from chemical ablation of the beta cells to breeding rodents that spontaneously develop autoimmune diabetes. During the last 25 years, two key animal models of type 1 DM – the inbred Bio Breeding (BB) rat and nonobese diabetic (NOD) mouse – have been used to study the genetics, pathophysiology, environmental impact, and treatment. NOD mice are characterized by insulitis. There is a pancreatic

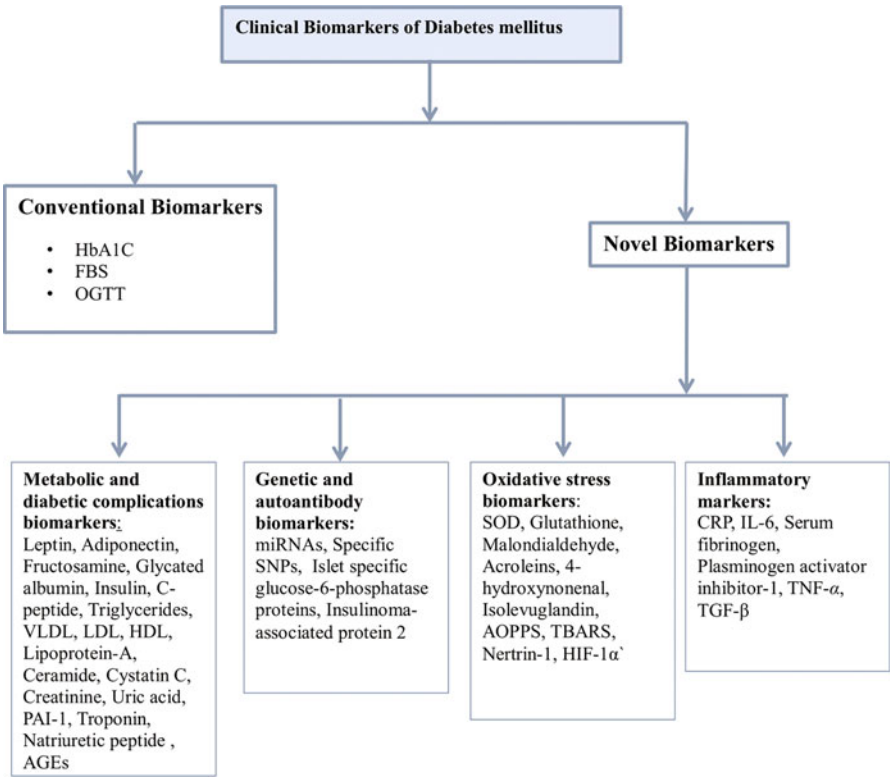

Fig. 2 Clinically used biomarkers in diabetes mellitus. *HbA1C* hemoglobin A1c, *FBS* fasting blood sugar, *OGTT* oral glucose tolerance test, *LDL* low density lipoprotein, *HDL* high density lipoprotein, *VLDL* very low density lipoprotein, *PAI-1* plasminogen activator inhibitor, *AGEs* advanced glycation end products, miRNA micro ribonucleic acid, *Specific SNPs* specific single nucleotide polymorphisms, *SOD* superoxide dismutase, *AOPPs* advanced oxidation protein products, *TBARS* thiobarbituric acid reactive substances, *HIF-1α* hypoxia-inducible factor-1α, *CRP* C-reactive protein, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor α, *TGF- β* transforming growth factor β. The conventional biomarkers are part of routine investigations in the diagnosis and monitoring the treatment of diabetes mellitus. The novel biomarkers are being validated through research

islet cell infiltration of $CD4^+$ and $CD8^+$ T cells, B lymphocytes, and variable numbers of macrophages/dendritic cells as early as 3 weeks postpartum. Hence it appears to be a suitable model for type 1 DM (Mordes et al. 2004).

The nongenetic models include the experimental models induced either by surgical removal or chemical destruction of pancreas using STZ or alloxan. Pancreatectomized animals particularly the dogs are used for studying type 1 DM (Kretschmer et al. 1977). High doses of Streptozotocin or alloxan have shown to induce an absolute deficiency of insulin, indicating type 1 or severe type 2 DM (Radenković et al. 2016).

Alloxan and STZ are glucose analogues that accumulate in pancreatic beta cells via the GLUT2 glucose transporter, causing toxicity. In the presence of intracellular

thiols such as glutathione, alloxan generates reactive oxygen species, which cause death of the pancreatic beta cells as they have a low antioxidant mechanism (Cohen and Heikkila 1974). STZ alkylates the beta cell membranes, fragments DNA, and thus destroys the beta cells. These agents at high doses cause an insulin-dependent diabetes state; however, at STZ induces type 2 DM at low doses. STZ also affects the beta cell mitochondrial function, thereby reducing the glucose-induced insulin secretion (LeDoux et al. 1986).

STZ is administered intraperitoneally or intravenously. Usually, chemically induced diabetes is carried out in rodent species, while few studies are also done in rabbits. STZ is preferred to alloxan for its extended stability, long-lasting diabetogenecity, higher inductive rate, lower toxicity, effectiveness across species, and less interference with the blood glucose monitoring. Mortality rate of animals is higher by alloxan than STZ due to an increased loss in body weight (Lenzen 2008).

Viruses such as Coxsackie virus injections can also be used to induce both type 1 and 2 DM. They bring about the destruction of pancreatic islets either by direct effects or by triggering an autoimmune response. Although used for both types of DM, they are preferred and used commonly for type 1 DM.

Some of the most commonly used models of type 1 DM are outlined in Table 1.

Table 1 Experimental models for Type 1 DM

Sl.No.	Model	References
Genetic	Akita diabetic mice	Fox et al. 2011
Spontaneous	Nonobese diabetic (NOD) mice BB (Biobreeding) rat LETL (Long-Evans Tokushima Lean) rat KDP (Komeda diabetes-prone) rat LEW.1AR1/Ztm-iddm	Mordes et al. 2004 Ikegami et al. 2004 Yokoi et al. 2003 Lenzen et al. 2001
Chemically induced	Streptozotocin-induced mouse model: A single, high dose of STZ (200 mg/kg) Multiple, low doses of STZ (40 mg/kg, intraperitoneally; i.p.) for 5 consecutive days	Wu and Huan 2008
	Streptozotocin-induced rat model: Single dose of STZ (40–70 mg/kg) to rats aged 8–10 weeks	Wu and Huan 2008
	Alloxan-induced mouse model: Single dose of 100–200 mg/kg BW	Siddiqui et al. 2014
	Alloxan-induced rats model: Single dose of 40–200 mg/kg BW	Ighodaro et al. 2017
Virus induced	Cox sackie virus infection Lymphocytic choriomeningitis virus (LCMV) H-1 parvovirus Kilham rat virus	King 2012

Experimental Models for Type 2 DM

The pathogenesis of type 2 DM involves both reduced insulin secretion as well as peripheral insulin resistance. The models are therefore developed to address both of these mechanisms.

Genetic models could be either monogenic with single dysfunctional gene such as Lep $^{ob/ob}$ mouse with defective Leptin gene or polygenic such as the inbred C57BL/6J mouse. The New Zealand Obese (NZO) mouse, TALLYHO/Jng mice characterized with varying levels of susceptibility to environmental factors.

Type 2 DM may also be induced chemically with STZ or alloxan. However, these chemicals have shown a high mortality when used alone and may not replicate many pathophysiological processes of human type 2 DM. Hence, the recent models have used a combination of high fat diet/sucrose/fructose with low dose STZ mimicking type 2 DM. High-fat diet (HFD) causes hyperinsulinemia and insulin resistance in peripheral tissues due to lipid toxicity. The low dose of STZ (30–40 mg/kg i.p) results in defective insulin secretion due to reduction in beta cell mass (Unger et al. 2010). Long-term administration of HFD has shown to induce hypoinsulinemia (Nadig et al. 2021). This model is sensitive to the glucose lowering effects of insulin sensitizers such as pioglitazone as well as insulinotropic agents such as glipizide. Features of dyslipidemia were also observed that were reversed with Pioglitazone and Glipizide (Srinivasan et al. 2005).

Virus-induced models using Cocksackie virus are also used for type-2DM. Most commonly used models for experimental type 2 DM are shown in Table 2.

Experiments with *Syzygium Cumini*

The successful induction of the desired type of experimental DM, and the response to therapy is measured using validated biomarkers. Most of the biomarkers in animal models are developed based on the clinical biomarkers currently used in the management of DM, as discussed in the previous section. The biomarkers used during the experiments with SC are described below.

Fasting Blood Glucose (FBG)

Hyperglycemia observed as an increase in the blood glucose is the key diagnostic feature of DM. Patients with type 2 DM exhibit a higher rate of hepatic glucose output due to insulin resistance as compared to healthy subjects, which is the main contributor to fasting hyperglycemia. Hence FBG is considered as an important biomarker used in experimental DM too.

Generally the FBG is measured between 48 h– and 7 days after induction of DM using glucose oxidase method. Levels above 200–300 mg/dl are considered as diabetic in any rodent species. The severity of induction of DM and the response to treatment depends on the inducing agent and its dose.

Table 2 Experimental models for Type 2 DM

Sl.No.	Model	References
Genetic	The KK mouse Obese hyperglycemic mice $Lep^{ob/ob}$ mouse C57BL/6J mouse strain The New Zealand Obese (NZO) mouse TALLYHO/Jng mice Zucker Diabetic Fatty rats	Kottaisamy et al. 2021
Spontaneous	Otsuka Long-Evans Tokushima Fatty Rat Goto-Kakizaki rats db/db (C57BL/KsJ-db/db) mice The Nagoya–Shibata–Yasuda (NSY) mouse Psammomys obesus (the Israeli sand rat) Spiny mouse	Rees and Alcolado 2005
Chemically induced	**Mouse Models**:	
	Single dose of Alloxan (50–200 mg/kg BW) Single dose STZ (100–200 mg/kg BW) STZ (50 mg/kg) – nicotinamide (120 mg/kg)	Siddiqui et al. 2014 Chatzigeorgiou et al. 2009 Birgani et al. 2018
	Rat Models:	
	Single dose of Alloxan (40–200 mg/kg BW) Single dose streptozotocin (35–65 mg/kg BW) HFD-STZ(40 mg/kg) HFD-fructose model High-fat chow for 4 weeks followed by STZ (35 mg/kg, i.p.) Monosodium L-glutamate- (MSG-) (4.0 g/kg/day) induced obese rats High carbohydrate, high fat diet (HFHS)-induced diabetic Wistar rats	Ajiboye et al. 2018 Vora et al. 2019 Nadig et al. 2021 Munshi et al. 2014 Chao et al. 2018 França et al. 2019 Ulla et al. 2017
Virus induced	Coxsackie B virus EMC virus	King 2012
Surgery	Pancreatectomy model	Kottaisamy et al. 2021

Studies with SC extracts have shown significant effects on FBG in both alloxan as well as STZ-induced diabetes models (Siddiqui et al. 2014). In a study by Sharma et al. with HFD-STZ, SC aqueous seed extract showed 38% reduction in FBG compared to 59% by metformin administered for a period of 21 days (Sharma et al. 2012). Hydroalcoholic extract of SC seeds in long-term HFD-STZ (40 mg/kg) model by Nadig et al. showed a reduction of 76% in FBS in comparison with pioglitazone that showed 70% reduction (Nadig et al. 2021).

Aqueous extract of EJ seed (400 mg/kg) given in combination with a DPP4 inhibitor, sitagliptin (10 mg/kg) for a duration of 28 days to STZ-induced diabetic rats showed a synergistic antidiabetic effect in reducing the FBG. In addition there was a significant improvement in the kidney function, the liver function, and the lipid parameters (Vora et al. 2019).

Serum Insulin and C Peptide Levels

Insulin, a peptide hormone released by beta cells of pancreas is mainly involved in regulating carbohydrate, protein, and fat metabolism. It regulates the glucose uptake in insulin-dependent tissues, facilitates cell division and growth (Wilcox 2005).

Generally, the normal range of insulin in rodents is observed to be in the range of 13–16 microIIU/ml. Decrease in plasma insulin levels in animal models after STZ application is used as a sign for induction of diabetes (Koksal 2015). However, in some experiments with low dose STZ-HFD or low dose STZ-fructose, hyperinsulinemia is observed (Sharma et al. 2012; Munshi et al. 2014). Such models usually indicate a prediabetic or early diabetic state. Majority of STZ models however have shown reduced insulin secretion. The effect size depends on the dose of the inducing agent and the time of measurement of plasma insulin (Koksal 2015).

In STZ-treated isolated pancreatic islets of rats, rutin and quercetin, important phytoconstituents in SC have shown an increase in insulin secretion through L-type of calcium channels, adiponectin and leptin (Chagas et al. 2015).

Purified fraction of SC (L.) Skeels (SC2), polyphenol rich leaves extract, ethanolic extract of seeds, and hydroethanolic extract of leaves have all shown an increase in the plasma insulin in various models using STZ, alloxan, and monosodium glutamate (Ajiboye et al. 2018; Sharma et al. 2011).

C peptide is a precursor of insulin and also an important parameter to measure the beta cell function and insulin secretion. C-peptide levels <0.2 nmol/l is associated with type 1 DM. In STZ (45 mg/kg)-induced diabetic male Wistar albino rat models, SC bark extract (300 mg/kg body weight) significantly elevated plasma insulin levels and C-peptide in comparison to Glibenclamide (Saravanan and Leelavinothan 2006).

Glycosylated Hemoglobin (HbA1C)

Glycosylated hemoglobin (HbA1C) often reflects chronic hyperglycemia and is a predictor of diabetic complications. HbA1C is formed when glucose imbibes into the β subunit of hemoglobin. ADA criteria of HbA1C is 5.7–6.4% (39–46 mmol/mol) for prediabetes and $\geq$6.5% (48 mmol/mol) for diabetes (ADA 2021).

In a study by Sherafeldin et al., HbA1C in the normal Wistar albino rats was found to be $7.02 \pm 0.17\%$. Induction of diabetes with STZ increased the levels to 12.07 ± 0.30, which was statistically significant. Administration of SC ethanolic seed extract, 200 mg/body weight once weekly for 4 weeks significantly reduced elevated HbA1C levels compared to diabetic control rats (Sharafeldin and Rizvi 2015). Daily administration of seed extracts has also shown similar reduction in HbA1C (Ravi et al. 2004).

In alloxan-induced mild diabetic (MD) and severely diabetic (SD) rabbits, purified fraction of ethanolic extract of SC seeds (LHI,II,III) were evaluated at a dose of 10 mg/ kg body weight for a duration of 7 days (MD) and 15 days (SD). LHII

fraction significantly resulted in 50.5% fall in HbA1C after 15 days of treatment (Sharma et al. 2011).

HbA1C is an indicator of long-term control of DM but limited literature is available in experimental diabetes model due to the short duration of studies.

Homeostatic Model of Assessment (HOMA)

Pancreatic β-cell dysfunction and insulin resistance (IR), the key pathognomonic features of type 2 DM, can be assessed from FBG and plasma insulin/C-peptide concentration. Homeostatic model of assessment IR (HOMA-IR) is a reliable surrogate biomarker of IR while HOMA β reflects the insulin secretory function of pancreatic beta cell (Wallace et al. 2004).

HOMA-β is given by the formula = (20 × insulin in mIU/mL)/ (glucose in mmol/L – 3.5).

HOMA-IR: fasting blood glucose (FBS) (mg/dL) × insulin (μIU/mL)/405. The normal HOMA-IR value of healthy human ranges 0.5–1.4. [<1.0 = optimal, >1.9 indicates early insulin resistance, and >2.9 indicates significant insulin resistance (Wallace et al. 2004).

IR and insulin signaling were compared in four models of type 2 DM: rats fed on a fructose-rich chow for 8 weeks, rats fed with high-fat chow for 4 weeks followed by injection with STZ (35 mg/kg, i.p.), rats injected with a single low dose STZ (45 mg/kg, i.p.), and rats injected with a single dose of nicotinamide followed by a single high dose of STZ (60 mg/kg, i.p). Among these four, HFD/STZ rats was found to be an appropriate and stable animal model for simulating IR analogous to the human T2 DM (Chao et al. 2018). This model also exhibited a reduction in HOMA β. Both were reversed to near normal levels with administration of aqueous or hydroalcoholic extract of seeds of SC (Sharma et al. 2017; Nadig et al. 2021).

In alloxan-induced diabetic rats, polyphenolic-rich extract of SC *(*400 mg/kg body weight: free phenol and bound phenol), HOMA-IR levels were significantly decreased and HOMA β levels increased with both forms of extract comparable to that of metformin and diabetic control (Ajiboye et al. 2018).

In all the above models, reduction in HOMA β appears to be due to destruction of pancreatic beta cells and increase in HOMA-IR as a result of increase in inflammation due to reactive oxygen species as a result of STZ and Alloxan administration.

Thus HOMA β and HOMA-IR appear to be important surrogate biomarkers to assess the improvement of beta cell function and insulin resistance in response to antidiabetic treatment.

Liver Glycogen

The synthesis and maintenance of glycogen stores are some of the very characteristic actions of insulin. Hepatic glycogenesis depends on glucose as substrate and insulin for the process of glycogen synthesis with the help of enzymes. In type 1 and type

2 DM, there is reduced glycogen synthesis as insulin levels are low. Even if the levels of insulin are normal or high (as it happens in the prediabetic or early diabetic stage), hepatic insulin resistance may result in reduced glycogen storage. This in turn results in increased hepatic glucose output accounting for hyperglycemia (Jiang et al. 2020).

Magalhães et al. demonstrated a reduction in liver glycogen from 30.2 to 8.5 in HFD-STZ (40 mg/kg)-induced type 2 DM in Wistar albino rats (Magalhães et al. 2019). Ravi et al. also showed more than 50% reduction in the liver glycogen content in STZ (55 mg/kg body weight)-induced diabetic Wistar albino rats compared to healthy controls, which was reversed when treated with aqueous seed and kernel extract of EJ administered orally for 30 days (Ravi et al. 2005). Similar increase in hepatic glycogen was observed in alloxan-induced diabetic rats with purified fractions of ethanolic seed extracts of SC in both mild and severely diabetic animals (Sharma et al. 2011). Hepatic IR induced by HFD appears to contribute for increase in liver glycogen.

Lipid Profile

DM is characterized by derangement in cholesterol and triglyceride levels. Deficiency of insulin or insulin resistance leads to uncontrolled dyslipidemia via mobilization of free fatty acids (FFA) increasing the HMG-CoA reductase activity and decreasing lipoprotein lipase (LPL) activity. Uncontrolled diabetic dyslipidemia is an important risk factor for atherogenic complications.

On administration of hydroethanolic extract of SC leaf (HESc) (0.5 g/kg/day and 1.0 g/kg/day) in monosodium L-glutamate-(MSG-) (4.0 g/kg/day) induced obese rats, there was reduction in weight gain by 15% and adipose tissue fat pads, FBG, triglycerides, total cholesterol, and free fatty acids, as well as insulin sensitivity similar to lean rats. The extract reduced fat accumulation in liver by 13% in 0.5 g/kg dose group and 27% in the 1 g/kg dose group. VLDL levels were reduced by 50% in both treated groups (França et al. 2019).

In STZ (45 mg/kg)-induced diabetic rats fed with atherosclerotic diet (1.5 ml olive oil containing 8 mg (3,20,000 IU) vitamin D_2 and 40 mg cholesterol) given for 5 consecutive days, active compound FIIc of FII fraction of lyophilized aqueous extract of SC pulp administered for 30 days reduced the levels of TG, TC, LDL-C, and VLDL-C and improved the levels of HDL in comparison glibenclamide. There was significant improvement in the levels of $ApoA_1$, $ApoB_{100}$, and $ApoB_{100}/ApoA_1$ ratio. $ApoB_{100}$ has been a significant factor to predict coronary artery disease (Tanwar et al. 2011).

In STZ (55 mg/kg)-induced diabetic rats, administration of ethanolic extract of EJs-kernel (100 mg/kg body weight) was able to significantly reverse dyslipidemia (elevated LDL, VLDL, decreased HDL) to normal levels (Chaturvedi et al. 2009).

The possible factors contributing to the antidyslipidemic properties of SC has been attributed to several factors, such as inhibition of HMG-CoA reductase activity in the liver, reduction in the absorption of cholesterol by the intestine, or increased TG and FFA clearance in the periphery by stimulation of LPL (Sharma et al. 2008).

Adiponectin

Adiponectin is a protein secreted by adipocytes that have a significant effect on plasma glucose and lipid levels. Serum levels of adiponectin decrease during obesity and are directly associated with insulin sensitivity. Excessive expression of adiponectin in the liver reduces ceramide levels and improves insulin sensitivity. In an experiment with gene therapy with adiponectin in high fat high sucrose fed mice, there was lower blood glucose and insulin levels, improved glucose tolerance, and reduced hepatic gluconeogenesis compared with control mice (Kandasamy et al. 2012).

In STZ (45 mg/kg of b.w.)-nicotinamide (230 mg/kg b.w. given 15 min prior to STZ injection)-induced type 2 DM rats, administration of antihyperglycemic compound obtained from the fruit pulp of *EJ showed an* increased serum adiponectin level at week 6 as compared to diabetic controls (Jafri et al. 2019).

In an in vitro study on mouse 3 T3-L1 preadipocytes and rat L6 myoblasts, administration of logarithmic doses of SC methanolic extract (SCME) and Vitalboside A (VBA) isolated from SC (1 ng-10 μg/mL) showed a significant elevation in adiponectin secretion. Hence, it is clear that VBA showed a partial transcriptional activation of PPARc2 with a simultaneous increase in the secretion of adiponectin correlating with the decreased lipid accumulation in adipocytes. VBA-mediated combined activation of PPAR γ-transactivated adiponectin and PI3K/Akt-stimulated adiponectin secretion contributed to alleviated development of type 2 DM, insulin resistance, and obesity (Thiyagarajan et al. 2016).

Effect of SC on adiponectin levels needs to be explored as there is limited number of studies.

Glucagon-Like Peptide 1 (GLP 1)

GLP 1 is an insulinotropic hormone released from the gastrointestinal tract in response to food and enhances the postprandial insulin secretion. It is metabolized by DPP-4(dipeptidyl peptidase 4). The drugs that inhibit DPP4 such as sitagliptin enhance insulin secretion and suppress glucagon secretion.

There are limited experimental studies of SC using glucagon and GLP 1 as biomarkers. An in-vitro (DPP-4 inhibition assay) and in-vivo (STZ-induced diabetic rats) study using a polyherbal formulation containing the semi-standardized extracts of *Pterocarpus marsupium*, *EJ*, and *Gymnema sylvestre* by Kosaraju et al. showed an increase in GLP1 levels, which was consistent with elevated insulin levels (Kosaraju et al. 2014).

More research needs to be done to establish the conclusive role of GLP-1 as biomarkers in animal models.

Markers of Oxidative Stress

Oxidative stress has been implicated in the causation of IR, β cell failure, its progression to DM, as well as its atherosclerotic complications. Oxidative damage

occurs as a result of glucotoxicity and lipotoxicity. It induces liberation of products of lipid and protein oxidation such as malondialdehyde, thiobarbituric acid reactive substances (TBARS), advanced glycation end products (AGEs), and advanced oxidation protein products (AOPP) (Fiorentino et al. 2013).

SC has been evaluated both in the in vitro assays and in vivo experimental diabetes models for the antioxidant activity. Administration of SC aqueous seed extract to HFD-STZ-induced diabetic Wistar albino rats showed a dose-dependent reduction in TBARS and increase in the levels of SOD, CAT, and GSH-Px activities in comparison to diabetic control rats (Sharma et al. 2012).

Oral administration of 2.5 and 5.0 g/kg body weight of the aqueous extract of the SC seeds for 6 weeks in alloxan-induced diabetic rats resulted in a significant reduction in TBARS, and increase in reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) clearly show the antioxidant property of the extract. The extract was more at 5.0 g/kg body weight and better than that of glibenclamide (Prince et al. 1998). Similarly the ethanol seed extract of *Eugenia jambolana* at a dose of 100 mg/kg body weight also has shown to decrease the levels of TBARS, increase the levels of reduced GSH, OD, and CAT in the plasma of the STZ-induced diabetic rat (Ravi et al. 2004).

Administration of active principle (FIIc) isolated from aqueous fruit pulp extract of *Eugenia jambolana* to STZ-induced diabetic models showed a protective effect by elevating the nitric oxide levels, reducing the levels of Ox-LDL, reversing the sVCAM-1 and fibrinogen levels to near normal in diabetic rats (Tanwar et al. 2011).

Administration of SC seed powder (20 g) to a high carbohydrate high fat diet (HCHF)-induced obese rats model for 56 days showed a significant reduction in plasma and liver malondialdehyde (MDA) concentrations, reduced the rise of APOP level when compared to HCHF group. SC seed powder supplementation significantly improved the levels of hepatic SOD, CAT activities, and restored the GSH level when compared to the control rats (Ulla et al. 2017).

Oxidative markers indicate underlying systemic inflammation in DM. They appear to be important biomarkers for screening of herbal extracts as many phytoconstituents have significant antioxidant activity. Hence it is recommended to use all of these biomarkers in the experimental models of DM.

Inflammatory Biomarkers

An elevation of inflammatory biomarkers in response to autoimmune beta cell destruction is observed in type 1 DM. The beta cell inflammatory reaction also results in abnormal cytokine production and the activations of pro-inflammatory signaling pathways.

TNF-α is a potent pro-inflammatory cytokine released from macrophages and T lymphocytes in type 1 DM with predominant role in the development of insulin resistance. TNF-α also has important pathophysiological role in development of microvascular complications as it is involved in growth stimulation, cytotoxicity, and angiogenesis. High levels of CRP have been reported to be a strong and independent predictor of myocardial infarction, ischemic stroke, type 2 DM, and hypertension.

Large-scale human prospective and genetic data provide evidence that IL-6-mediated inflammation is implicated in the etiology of T2DM (Dorcely et al. 2017).

In alloxan (150 mg/kg)-induced diabetic rats, administration of polyphenolic-rich extract of SC leaves (400 mg/kg free phenol, 400 mg/kg bound phenol) had significantly higher anti-inflammatory activity. The levels of IL- 6 and TNF-α decreased in comparison to diabetic + metformin groups and diabetic control group (Ajiboye et al. 2018).

Reddy et al. studied the role of polyherbal formulation containing a high fraction of powdered EJ seeds (20 g) at doses of 250 and 500 mg/kg BW administered for 16 weeks in STZ-induced diabetic nephropathy model in rats. There was a significant rise in IL-6, TGF-β, and TNF-α in the diabetic animals as compared to the normal controls which were significantly reduced in the treated group. These inflammatory markers were measured using ABCAM ELISA Kits (Reddy et al. 2019).

When rat heart-derived H9C2 cardiomyoblast cells were treated with methanol extract of SC seeds, the levels of proinflammatory cytokines such as TNF-α and IL-6 decreased suggesting that the extract is able to prevent the onset of glucose-induced cardiac inflammation (Atale et al. 2021). Figure 3 shows the role of inflammation and oxidative stress observed in experimental diabetes.

Histopathology of Islets of Pancreas

HFD followed by STZ or STZ alone induces oxidative damage to the pancreatic cells particularly the islets and distorts the architecture of pancreas. Suman et al. showed damaged islets of Langerhans, atrophy of beta cells, and reduced beta cell mass as compared to normal control in an HFD-STZ (40 mg/kg ip)-induced diabetes model in Wistar albino rats (Suman et al. 2016).

In yet another study on alloxan (60 mg/kg)-induced diabetes in rats, administration of ethanolic extract of SC seeds (750 mg/kg) reversed the alloxan-induced changes in alpha and beta cells such as derangements, clumping, degranulation, and hydropic degeneration in comparison to diabetic control (Singh and Gupta 2007).

An oral administration of SC aqueous extract of the bark administered for 30 days at doses of 1 g/kg rat body weight in alloxan induced diabetes has showed regeneration of beta cells pancreas (Schossler et al. 2004).

In a study by Sharma et al. 2012, HFD-STZ-induced moderate injury(++) to β cells of pancreas, which was characterized by moderate decrease in the amount of β-cells, moderate number of inflammatory cells, and moderate swelling of islet cells. Administration of SC seed extract (400 mg/kg dose) showed a complete reversal of these changes in diabetic rats (Sharma et al. 2012).

PPARs (Peroxisome Proliferator-Activated Receptors)

PPARs (peroxisome proliferator-activated receptors) are ligand-activated transcription factors of nuclear hormone receptor superfamily comprising of the following

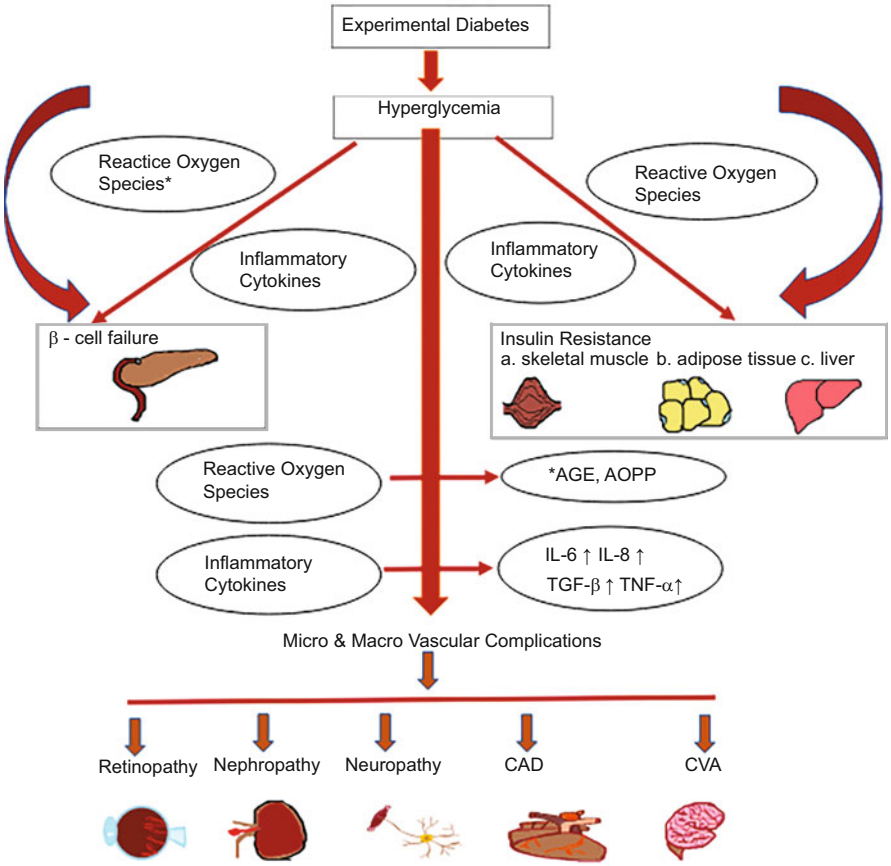

Fig. 3 Role of inflammation and oxidative stress in experimental diabetes. *IL-6* interleukin-6, *IL-8* interleukin-8, *TNF-α* tumor necrosis factor α, *TGF- β* transforming growth factor β, *CAD* coronary artery disease, *CVA* cerebrovascular accident, *AGE* advanced glycated end products, *AOPP* advanced protein oxidation products, *Reactive oxygen species (ROS)-Glutathione, Malonedialdehyde (MDA), Superoxide dismutase (SOD), Thibarbituric acid substances (TBARS), Catalase. Hyperglycemia in experimental diabetes results in the generation of reactive oxygen species, which leads to oxidative stress in beta cells of islets and the peripheral tissues. This is followed by the release of inflammatory cytokines such as TNF-α, IL-6, IL-8, TGF- β, etc. The resulting inflammation leads to beta cell dysfunction and insulin resistance. Uncontrolled hyperglycemia also leads to generation of other ROS such as AGE and AOPP that mediate the micro- and macrovascular complications due to vascular inflammation and endothelial dysfunction

three subtypes: PPARα, PPARγ, and PPARβ/δ. PPARγ distributed in adipose tissue, skeletal muscle, liver, and pancreatic islets play a key role in regulating the insulin sensitivity, adipocyte differentiation, inflammation, and cell growth (Semple et al. 2006). Various clinical studies have shown less expression of PPARγ protein in patients with type 2 DM.

Studies by Sharma et al. have shown that in HFD-STZ model, insulin resistance, dyslipidemia, and tissue damage is also accompanied by lowered expression of PPARγ in diabetic rats as compared with the normal control. SC extract was found to increase the hepatic PPARγ and PPAR α protein expressions after treatment at 400 mg/kg (Sharma et al. 2012).

Gallic acid is an important phytoconstituent of SC. Gandhi et al. have shown that gallic acid significantly enhances the level of peroxisome proliferator-activated receptor γ (PPARγ) expression in the adipose tissue of treated group compared to diabetic group and slightly activates PPARγ expressions in the liver and skeletal muscle (Gandhi et al. 2014).

Another study found that function of beta cells was significantly reduced (5.8-fold) in rats treated with a HFD-STZ which improved with aqueous seed extract of SC (400 mg/kg/day) and metformin. Through overexpression of PPARγ and PPARα activity, the aqueous extract of SC seeds exhibited significant insulin sensitizing, antidyslipidemic, antioxidant, anti-inflammatory, and beta-cell salvaging activity in HFD-STZ-induced type 2 diabetic rats, confirming its potential for use in the prevention and treatment of type 2 DM (Sharma et al. 2017).

Thus PPAR γ expression can be considered as an important biomarker indicating IR in the liver, skeletal muscle, adipose tissue, and beta cells.

Hexokinase

Hexokinase is a phosphorylating enzyme that exists in tissues in four isoenzymic forms. Type II hexokinase, which is known to be insulin-sensitive, is found predominantly in adipose tissue and mammary gland, type IV most often known as glucokinase is expressed primarily in the liver (Ali et al. 1980). It is an important enzyme required for glycolysis (Postic et al. 1994).

Expression pattern of Hex-1 in whole hepatic lysate as measured by RT PCR technique was reduced under diabetic settings compared to the control, which was reversed with ethyl acetate extract of seeds administered for 35 days of similar to previous findings that supported the hypothesis that higher levels of Hex1 activity reflect a higher rate of glucose utilization by cells (Jana et al. 2015).

Grover et al. observed that the aqueous and alcoholic extracts of the lyophilized powder of EJ partially restored the hepatic glucokinase, glucose-6-phosphate, hexokinase, phosphofructokinase levels, and the glycogen content in the liver and skeletal muscle in experimentally induced diabetic mice (Grover et al. 2000).

In yet another study by Sharma et al. glucose homeostatic enzymes such as glucose-6-phosphatase, hexokinase activities showed considerable improvement in STZ (4 mg /0.5 ml/100 mg body weight and 7 mg/0.5 ml/100 mg body weight)-induced diabetic rats after receiving flavonoid rich extract of SC seed (300 mg/kg/day, 15 days) as compared to diabetic counter parts (Sharma et al. 2008).

Biomarkers for Evaluating Molecular Mechanisms

Experiments have demonstrated that there are molecular alterations in the insulin-responsive tissues in the diabetic rats similar to those observed in human type 2 DM. Altered PI3K/AKT signaling pathway is shown to contribute to the development of IR in type 2 DM. There is a decreased expression of GLUT-4 receptors, insulin responsive substrate (IRS), or AKT pathway in insulin responsive tissues in type 2 DM (Huang et al. 2018).

Study by Rathinam et al. showed that in STZ-induced DM (40 mg/kg body weight) along with an increase in the levels of plasma glucose and decreased plasma insulin, there was a downregulation of insulin receptor substrate 2 (IRS2), AKT, and glucose transporter 2 (GLUT2) in liver and insulin receptor substrate 2 (IRS2), AKT, and glucose transporter 4 (GLUT4) protein expression in skeletal muscle (Rathinam and Pari 2016).

Long-term HFD-STZ-induced type 2 DM in Wistar albino rats showed an increase in FBG, decrease in plasma insulin, increased HOMA-IR, and decreased HOMA β along with decreased an expression of GLUT-4 receptor gene in skeletal muscle in diabetic rats. All these changes were reversed with the administration of hydroalcoholic seed extract SC 200 mg/kg body weight (Nadig et al. 2021).

An in vitro study of methanolic extract of SC extract on isolated L6 myotubes showed an increase in Glut-4, PPAR gamma, and PI3 kinase mRNA expression by semiquantitative RT-PCR. The elevated Glut-4 transcripts were comparable with insulin and rosiglitazone (Anandarajan et al. 2006).

Molecular Docking Study of *Syzygium Cumini* extract Components on Sulfonylurea Receptor (SUR1)

Computational methods are handy in understanding molecular level details such as interaction patterns of inhibitors and probable underlying mechanism of inhibition process. Molecular docking is one of such tools appreciated widely and well accepted by the scientific community both in academic and industrial research setup. The recent revolution of artificial intelligence/machine learning (AI/ML methods in several processes including biology and chemistry related approaches, structured-based molecular docking still enjoys its share in these processes and plays a vital support role to validate artificial intelligence/Machine learning (AI/ML) outcomes in drug discovery. Assan Aliyar et al. studied the molecular docking of gallic acid and ellagic acid with Sulfonylurea receptor 1 (SUR1) as they were important constituents of hydroalcoholic seed extract of *Syzygium cumini* (Assan Aliyar et al. 2021).

Analyses of gallic acid and ellagic acid-binding modes revealed that these compounds interact in a similar fashion like that of the reference compounds (repaglinide and glibenclamide). The carboxyl group of gallic acid forms hydrogen bond interaction with R1246 and is similar to repaglinide, whereas hydroxyl group of the third and fourth position forms hydrogen bond interaction with F433 main

chain carbonyl group. The phenyl ring forms hydrophobic interactions with side chains of I381, W430, F433, L434, and Y377 residues. Ellagic acid, a dimeric form of gallic acid, also forms hydrogen bond interaction with R1246 and demonstrated hydrogen bond interactions with the F433 main chain carbonyl group. Ellagic acid shows several stacking and hydrophobic interactions with hydrophobic residues (I381, W430, F433, L434, and Y377).

These molecular docking studies explained that both gallic and ellagic acid bind to the same site as that of the repaglinide site in SUR1/pancreatic ATP-sensitive K+ channel structure and make similar molecular interactions in the active site.

Thus, it appears that the insulin secretion activity observed for SC extract may be due to the binding of gallic and ellagic acids to SUR1 and thus inhibition of pancreatic ATP-sensitive K+ channel.

Table 3 summarizes the biomarkers studied on the experiments with SC.

Applications to Prognosis

In the present chapter, we have described the biomarkers in experimental diabetic models that reflect the underlying pathology in the organs involved in DM. For example, fasting blood glucose reflects pancreatic beta cell dysfunction as well as hepatic insulin resistance; PPAR γ and GLUT 4 expression in the adipose tissue/skeletal muscle reflect the peripheral insulin resistance; TBARS and SOD in the blood represent the systemic oxidative stress; TNF alpha and CRP reflects inflammatory state of the body. Furthermore, they are detected and measured both in the blood as well as the tissue homogenates.

Similar biomarkers are detected in humans and utilized for monitoring the clinical improvement. They are mostly the blood markers though few markers are derived from biopsy specimens in the research settings. Among the several biomarkers researched clinically, FBS/PPBS OGTT and HbA1C are generally used as biomarkers for both diagnosis and evaluating the progression of DM. Sometimes plasma C peptide, and lipid profile is added to this panel. This seems to be inadequate for successful management of DM. The type and the levels of biomarkers indicate the severity of diabetes. They are also predictive of complications, e.g., Hs CRP for cardiovascular complications; measuring the TNF α for diabetic nephropathy. Detection of certain oxidative biomarkers in prediabetics may prevent development of DM too. Many research papers are published on validation of biomarkers clinically, but not being recommended. Including a panel of biomarkers in the list of investigations at regular intervals during the treatment of DM may help in predicting and preventing the complications in DM.

Mini Dictionary of Terms

Gene expression: The process by which information from a gene is used in the synthesis of a functional product such as a protein, which results as the final effect.

Table 3 Summary of antidiabetic effects of SC: Biomarkers studied

Part of the plant	Nature of extract	Model, species, and dose of the inducing agent	Duration of treatment with SC	Biomarkers studied	References
Seed	Aqueous extract	HFD-STZ, high-fat diet/streptozotocin-induced (HFD-STZ) diabetic rats, HFD (55% of calories as fat) STZ (40 mg/kg, i.p.)	21 days	Serum glucose, insulin, IR, TNF-α, dyslipidemia, pancreatic thiobarbituric acid-reactive substance PPARγ and PPARα protein	Sharma et al. 2012
Seed	Powder	High carbohydrate high fat diet (HCHF)-induced obese rats model	56 days	Serum blood glucose, hepatic SOD, CAT activities and restored the GSH level	Ulla et al. 2017
Seed	Betulinic acid extracted from seed	STZ-nicotinamide induced, Wistar rats, 55 mg/kg STZ by IP route, 100 mg/kg nicotinamide	28 days	Fasting blood glucose, body weight, LDL, VLDL, triglycerides, HDL, total cholesterol	Birgani et al. 2018
Seed	Hydro alcoholic extract	HFD-STZ (40 mg/kg)	21 days	Fasting blood glucose, HOMA-IR, HOMA-B, GLUT 4	Nadig et al. 2021
Seed	Aqueous extract	High fat-STZ induced, 40 mg/kg STZ by IP route	21 days	Serum glucose, insulin, HOMA-IR	Sharma et al. 2017
Seed	Ethanol extract	STZ-induced, Wistar Albino rats, 60 mg/kg STZ by IP route	28 days	Fasting blood glucose, total cholesterol, triglycerides, LDL-cholesterol HDL-cholesterol	Sharafeldin and Rizvi 2015
Seed	Ethyl acetate extract	STZ-induced, Wistar Albino rats, 40 mg/kg STZ by IP route	35 days	Serum insulin, glycated hemoglobin	Jana et al. 2015
Seed	Ethanolic extract	STZ-induced, CF Albino rats, 45 mg/kg STZ by IP route	10 days	Fasting blood glucose, serum cholesterol, triglycerides, insulin level, glycosylated hemoglobin	Chaturvedi et al. 2009

(continued)

Table 3 (continued)

Part of the plant	Nature of extract	Model, species, and dose of the inducing agent	Duration of treatment with SC	Biomarkers studied	References
Seed	Flavonoid-rich extract	STZ-induced, Wistar Albino rats, 7 mg/0.5 ml of physiological saline/100 g body weight (bw) by IP route	21 days	Fasting blood glucose (FBG), peak blood glucose level insulin, LDL, triglycerides, HDL	Sharma et al. 2008
Seeds	Ethanolic Extract	Alloxan-induced rats, Wistar albino rats, 150 mg/kg	15 days	Fasting blood glucose, histopathology of islets	Singh and Gupta 2007
Seed	Ethanolic extract	Alloxan-induced, Albino rabbits, 80 mg/kg STZ by IP route	21 days	Serum total cholesterol, triglycerides, high-density lipoprotein cholesterol, total cholesterol/high-density lipoprotein cholesterol	Sharma et al. 2011
Seed	Aqueous extract	Alloxan-induced diabetic rats		Serum blood glucose, GSH, SOD, CAT	Prince et al. 1998
Fruit	Triterpenoid-enriched extract	STZ-induced, C57BL/6 mice, 40 mg/kg STZ by IP route	14 days	Fasting blood glucose, Akt phosphorylation levels, GLUT 4 protein	Li et al. 2017
Fruit pulp	Aqueous extract	STZ-induced, Wistar Albino rats, 45 mg/kg STZ by IP route	14 days	Fasting blood glucose, **HbA1C**, body weight	Tanwar et al. 2011
Leaves	Polyphenol-rich extract (acetone extract)	Alloxan-induced, Wistar rats, 150 mg/kg alloxan by IP route	14 days	Fasting blood glucose, glycated hemoglobin levels, glucose-6-phosphatase activity, glycogen, insulin, antioxidant enzymes, hexokinase, IL6, TNFα	Ajiboye et al. 2018
Bark	Bark extract	STZ-induced, Wistar Albino rats, 50 mg/kg STZ by IP route	45 days	Fasting blood glucose, serum insulin, C peptide	Saravanan and Leelavinothan 2006

Genomics: Study of the genetic complement of an organism (the genome). Particular gene of interest can also be studied.

Glycosylated Hemoglobin: Hemoglobin to which glucose is bound. It increases in response to high blood sugar

Homeostatic model of assessment (HOMA): A method for assessing pancreatic islet β-cell function and insulin resistance (IR) from basal (fasting) glucose and insulin or C-peptide concentrations.

Insulin resistance: Resistance by the peripheral tissues such as muscle or adipose tissue in the insulin-mediated uptake of glucose

Oxidative stress: A disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses.

Pancreatic Beta cell dysfunction: Altered function of the beta cells of islets of pancreas that result in reduced insulin secretion.

Proteomics: Study of protein expression in the cells

Sulfonylurea receptor: Membrane proteins on the beta cells of pancreas, which are the molecular targets of the sulfonylurea class of antidiabetic drugs that release insulin from the pancreatic beta cells.

Key Facts: Biomarkers in Experimental Diabetes: Studies with *Syzygium Cumini* (L.) and Links with the Sulfonylurea 1 Receptor

- Pancreatic beta cell dysfunction, insulin resistance, and inflammation are the key features of DM.
- Newer treatments for DM are evaluated on experimental diabetic models that closely mimic the human diabetes using mostly the rats and mice.
- Among all the genetic and induced models of experimental diabetes, a combination of high fat diet-low dose Streptozotocin is the most preferred model for type 2DM.
- Fasting blood glucose, glycosylated hemoglobin, oral glucose tolerance test, and plasma lipids are most commonly used and validated biomarkers in diabetic animals used in evaluating the response to treatment.
- In addition, in type 1 DM models, auto antibodies, CD4 and CD8 lymphocyte count, are used to evaluate the autoimmune destruction of beta cells of islets of pancreas.
- The newer biomarkers are HOMA-IR, HOMA- β, PPARγ, plasma insulin, oxidative stress markers, and inflammatory markers.
- *Syzygium cumini* is an antidiabetic medicinal plant that improves insulin secretion (HOMA- β) by the pancreatic beta cells combining with the sulfonylurea receptor.
- It also reduces the insulin resistance (HOMA-IR) in the adipose tissue, skeletal muscle, and the liver through increased expression of PPAR γ, AKT pathways, and GLUT-4 receptors.
- The plant shows promising antidiabetic effects that have a potential for human use.

Summary

- Animal models for experimental diabetes are used to study the pathogenesis and evaluate the efficacy of new treatments.
- Among the various models available for type 1.DM, the most commonly used ones are the genetic models such as inbred Bio Breeding (BB) rat and nonobese diabetic (NOD) mouse and STZ/alloxan-induced DM. These models have demonstrated significant beta cell dysfunction.
- Commonly used models for Type 2DM are high fat diet-Low dose streptozotocin-induced models that have demonstrated decreased pancreatic beta cell function, insulin resistance, oxidative stress, and inflammation.
- The key biomarkers that are studied for pancreatic beta cell dysfunction include fasting blood glucose, plasma insulin levels, HOMA- β, GLUT-2 expression in the pancreatic islets and histopathology of islets of pancreas.
- Fasting blood glucose, HOMA-IR, plasma lipids, PPAR γ expression in the liver, skeletal muscle and adipose tissue, GLUT 4 expression in the skeletal muscle and adipose tissue, hepatic glycogen, hepatic hexokinase, glucose 6 phosphatase, and phosphofructokinase are some of the biomarkers utilized to indicate insulin resistance.
- Oxidative stress markers such as MDA, TBARS, SOD, catalase, etc., and inflammatory cytokines such as TNF-α are the newer biomarkers whose levels correlate with both beta cell failure and insulin resistance.
- AGE and AOPP are biomarkers used in experimental models of diabetic complications.
- *Syzygium cumini* also called *Eugenia jambolana* is an antidiabetic medicinal plant that has shown efficacy in both type 1 and type 2 experimental diabetes mellitus.
- Among all the parts studied with different solvent extracts, the hydroalcoholic extract of seeds of SC appears to be the most effective.
- The extracts have shown significant effects on almost all the biomarkers reviewed. The characteristic beneficial effect has been that of restoring the beta cell function observed as beta cell regeneration in the histopathological studies of pancreas.

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Part VIII

Resources

Recommended Resources for Biomarkers in Diabetes: Methods, Discoveries, and Applications

55

Rajkumar Rajendram, Daniel Gyamfi, Vinood B. Patel, and Victor R. Preedy

Contents

Introduction 1142
Resources 1143
Other Resources 1143
Summary Points 1148
References 1153

Abstract

Diabetes mellitus is a treatable disease characterized by persistent hyperglycemia. Its prevalence is spiraling out of control, resulting in significant morbidity and mortality worldwide. The early diagnosis of diabetes can reduce the risk of complications. The use of biomarkers may facilitate this. Indeed, several biomarkers are currently used in routine clinical practice. Yet, these have limitations, and novel biomarkers have been developed using "omic" technologies and other analytical platforms. Thus, the knowledge and awareness of this

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subject has developed in recent years. It is challenging even for well-established researchers to keep abreast of research related to biomarkers of diabetes or to employ those resources used or recommended by other experts or practitioners in the field. To assist, we have compiled a list of resources as recommended by active practitioners and researchers. These tables include information on regulatory bodies, societies, other organizations, books, journals and other resource materials of practical use.

Keywords

Books · Evidence · Journals · Development · Professional societies · Regulatory bodies

Introduction

Diabetes mellitus is a treatable disease characterized by persistent hyperglycemia. This complex metabolic disorder still causes significant morbidity and mortality worldwide. Its prevalence is spiraling out of control. The global prevalence of diabetes mellitus is currently estimated to be over 500 million. However, by 2045, it is predicted that this could exceed 780 million (International Diabetes Federation 2017, 2022).

Biomarkers can be defined as "characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention" (Atkinson et al. 2001). Biomarkers have significant value in the study and treatment of diabetes mellitus.

Diagnosing diabetes at an early stage can reduce individuals' risk of developing complications (Dorcely et al. 2017). This may be facilitated by the use of biomarkers. Indeed, several biomarkers are currently used in routine clinical practice by physicians specializing in the management of patients with diabetes mellitus (Dorcely et al. 2017; Ahluwalia et al. 2019). These include glycated albumin and fructosamine (Dorcely et al. 2017). However, the most commonly used biomarkers of diabetes mellitus are blood glucose and glycated hemoglobin (HbA1c) (Dorcely et al. 2017). Regardless, all of these biomarkers have limitations (Dorcely et al. 2017; Ahluwalia et al. 2019). Their sensitivity and specificity are reported to be suboptimal (Dorcely et al. 2017).

Therefore, additional biomarkers have been sought. Omic technologies (e.g., genomics, and proteomics) and other analytical platforms have been used to identify several potential biomarkers of diabetes mellitus (Ahluwalia et al. 2019). Yet each individual biomarker has limitations. Thus, combining biomarkers may better identify those at risk of developing diabetes and its complications (Dorcely et al. 2017). In future, "personalisation" of disease

management may involve the use of emerging high-throughput biomarker technologies.

Even experienced scientists, researchers, and clinicians struggle to stay up-to-date all to use the most suitable resources. To address this, we have compiled tables containing resources as used and recommended by active researchers and practitioners who specialize in either diabetes and/or biomarker discovery. These resources are designed to assist colleagues who are interested in the use of biomarkers and their application to diabetes in both the preclinical and clinical settings. Essentially, the tables included herein draw upon the experiences and acumen acquired over many years: in some cases, decades. The list below acknowledges all the experts who helped to prepare these valuable resources.

Resources

Tables 1, 2, 3, 4, 5 and 6 list the most up-to-date information on the regulatory bodies (Table 1), professional societies (Table 2), journals (Table 3), books (Table 4), emerging technologies, and platforms (Table 5), and other resources of interest (Table 6) that are relevant to an evidence-based approach to biomarkers, diabetes, or the combination of both these scientific domains. Some organizations are listed in more than one table as they occasionally fulfill multiple roles.

Other Resources

Both the Wellcome Collection (https://wellcomecollection.org/collections) and The British Library (https://www.bl.uk/) also list material on topics related to analytical sciences and medicine, including diabetes. The National Library of Medicine (https://www.nlm.nih.gov/) is also a useful resource.

Other chapters on resources (recommended by authors and practitioners) may also be relevant to biomarkers of diabetes. These include diabetes and oxidative stress (Rajendram et al. 2020), maternal diabetes (Rajendram et al. 2017a, b), general aspects of biomarkers (Rajendram et al. 2016a), biomarkers of cardiovascular disease (Rajendram et al. 2016b), biomarkers of renal disease (Rajendram et al. 2017b), aging (Rajendram et al. 2021), diet and nutrition in critical care (Alzaid et al. 2015), and the metabolism and physiology of bariatric surgery (Rajendram et al. 2016c).

This list of material in these tables is included to provide general information only. It does not constitute any recommendation or endorsement of the activities of these sites, facilities, or other resources listed in this chapter, by the authors or editors of this book.

Table 1 Regulatory bodies, groups, and organizations dealing with biomarkers or diabetes

Regulatory body, organisation or group	Web address
American Academy of Ophthalmology	https://www.aao.org/
Biomarkers and Diagnostic Testing community – Johns Hopkins Institute for Clinical and Translational Research	https://ictr.johnshopkins.edu
Biomarkers Consortium – Foundation for the National Institute of Health	https://www.fnih.org
Biospecimen Exchange for Neurological Disorders (BioSEND)	https://biosend.org
Diabetes Atlas – International Diabetes Federation	https://www.diabetesatlas.org
Food and Drug Administration	https://www.fda.gov
International Diabetes Federation (IDF)	https://idf.org/
International Federation of Clinical Chemistry – IFCC	https://www.ifcc.org/
Internationally Peer Reviewed Chemical Safety Information (INCHEM) – World Health Organization	https://inchem.org/
Juvenile Diabetes Research Foundation (JDRF)	https://www.jdrf.org/
National Eye Institute (NEI) – NIH	https://www.nei.nih.gov/
National Glycohemoglobin Standardization Program	http://www.ngsp.org/
National Health Service (UK)	https://www.nhs.uk/
National Institute of Diabetes and Digestive and Kidney Diseases	https://www.niddk.nih.gov
National Institute of Neurological Disorders and Stroke – NIH	https://www.ninds.nih.gov/
National Institute of Standards and Technology	https://www.nist.gov/
National Medical Products Administration, China	http://english.nmpa.gov.cn/
The Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC)	https://magicinvestigators.org/
United States Food & Drug Administration	https://www.fda.gov/
World Health Organization (WHO)	https://www.who.int/

This table lists the regulatory bodies, groups, and organizations involved with biomarkers or diabetes. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers or diabetes within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 2

Table 2 Professional societies relevant to biomarkers or diabetes

Society name	Web address
Alzheimer's Association	https://www.alz.org/
American Association for Clinical Chemistry (AACC)	https://www.aacc.org/
American Diabetes Association	https://www.diabetes.org/
American Society for Nutrition	https://nutrition.org/
American Society of Human Genetics (ASHG)	https://www.ashg.org/
Biomarkers Study Group of the Association for Acute CardioVascular Care – ESC	https://www.escardio.org/Sub-specialty-communities/Association-for-Acute-CardioVascular-Care-(ACVC)/About/Biomarkers-Study-group
European Association for the Study of Diabetes	https://www.easd.org/
European Society of Cardiology (ESC)	https://www.escardio.org/
European Society of Radiology	https://www.myesr.org/
International Society of Antimicrobial Chemotherapy – Rapid Diagnostics & Biomarkers	https://www.isac.world
International Society of Oncology and Biomarkers	https://www.isobm.org
National Multiple Sclerosis Society	https://www.nationalmssociety.org/
Türk Diabet Cemiyeti	https://diyabetcemiyeti.org/
Understanding Society	https://www.understandingsociety.ac.uk/

This table lists the professional societies involved with biomarkers or diabetes. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers or diabetes within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 1

Table 3 Relevant journals publishing original research and review articles related to biomarkers of diabetes

Acta Diabetologica
Atherosclerosis
Biomedicine and Pharmacotherapy
BMC Cardiovascular Disorders
BMC Endocrine Disorders
Cardiovascular Diabetology
Cells
Diabetes
Diabetes and Metabolic Syndrome: Clinical Research and Reviews
Diabetes and Vascular Disease Research
Diabetes Care
Diabetes Metabolism Research and Reviews
Diabetes Research and Clinical Practice
Diabetologia
Frontiers in Endocrinology
Frontiers in Immunology
International Journal of Molecular Sciences
Journal of Clinical Endocrinology and Metabolism
Journal of Clinical Medicine
Journal of Diabetes
Journal of Diabetes and Its Complications
Journal of Diabetes Investigation
Journal of Diabetes Research
Journal of Diabetes Science and Technology
Journal of the American Heart Association
Nutrients
Nutrition Metabolism and Cardiovascular Diseases
Plos One
Primary Care Diabetes
Scientific Reports

Journals publishing original research and review articles related to biomarkers of diabetes. Included in this list are the top 30 journals which have published the most number of articles on the biomarkers of diabetes over the past 5 years. Data derived from Scopus

Table 4 Books on biomarkers or diabetes

Book title	Authors or editors	Publisher	Year of publication
Analysis of Biomarker Data: A Practical Guide	Looney SW, Hagan JL	John Wiley & Sons	2015
Biomarkers and Biosensors	Sadana A, Sadana N	Elsevier Science	2014
Biomarkers in Health and Disease: Further Knowledge	Rajendram R, Rajendram R, Patel VB, Preedy VR	Springer	2015
Biomarkers of Disease: An Evidence-Based Approach – first Edition	Trull AK	Cambridge University Press	2008
Biomarkers of Environmentally Associated Disease: Technologies, Concepts, and Perspectives	Suk WA	CRC Press	2002
Biomarkers of Neurological and Psychiatric Disease, Volume 101	Bahn S, Guest P	Academic Press	2011
Biomarkers: In Medicine, Drug Discovery, and Environmental Health	Vaidya VS, Bonventre JV	John Wiley & Sons	2010
Clinical Management of Shock – The Science and Art of Physiological Restoration	Stawicki SP, Swaroop M	IntechOpen	2020
Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation	World Health Organization	Geneva: World Health Organization	2006
Diabetes Mellitus: Impact on Bone, Dental and Musculoskeletal Health – first Edition	Tan M	Elsevier	2020
Endocrine Biomarkers	Sadrzadeh H, Kline G	Elsevier	2017
Epigenetic Biomarkers and Diagnostics first Edition	García-Giménez JL	Academic Press	2015
Epigenetic Biomarkers and Diagnostics	Garcia-Gimenez JL	Academic Press	2015
Genomic Biomarkers for Pharmaceutical Development: Advancing Personalized Health Care	Yao Y, Jallal B, Ranade K	Academic Press	2013
Inflammatory Pathways in Diabetes: Biomarkers and Clinical Correlates	Pugia M	Springer	2015
International Diabetes Federation Diabetes Atlas – ninth Edition	International Diabetes Federation	Brussels, Belgium: International Diabetes Federation	2019

(continued)

Table 4 (continued)

Book title	Authors or editors	Publisher	Year of publication
Novel Biomarkers to Understand Cardiovascular Complications in Diabetes	Adela R, Banerjee SK	IntechOpen	2016
Nutrition and Diet in Maternal Diabetes	Rajendram R, Preedy VR, Patel VB	Springer	2018
Proteomic and Metabolomic Approaches to Biomarker Discovery	Issaq H	Academic Press	2013
Proteomics: Methods and Protocols	Reinders J, Sickmann A	Springer	2009
The Detection of Biomarkers – first Edition	Ozkan S, Bakirhan N, Mollaraosouli F	Academic Press	2021
The Diabetes Textbook	Rodriguez-Saldana J	Springer	2019
The Handbook of Biomarkers	Jain KK	Humana Press	2017
Type 1 Diabetes: Pathogenesis, Genetics and Immunotherapy	Wagner D	InTech Open	2011

This table lists books relevant to biomarkers or diabetes

Summary Points

- Biomarkers of diabetes mellitus have significant clinical value in modern medicine.
- Biomarkers can be used in the screening for diabetes mellitus.
- Biomarkers can be used to direct therapy after the diagnosis of diabetes.
- Biomarkers can be used to monitor the response to treatment and guide the choice of further treatments for diabetes.
- This chapter lists the most up-to-date resources relevant to the use of biomarkers of diabetes.

Table 5 Techniques and platforms related to biomarkers or diabetes

Organization or company name	Web address
Active Motif	https://www.activemotif.com/
Bevital AS	https://bevital.no/
Biomarker and Genomic Sciences Group	https://www.nist.gov/mml/bbd/bioassay-methods
Biomarker Solutions – Labcorp	https://drugdevelopment.labcorp.com/services/clinical-testing/precision-medicine-solutions/biomarker-solutions.html?utm_source=google&utm_medium=cpc&utm_campaign=G_Nbr_Covance+Central+Labs+%5BBEP%5D%3BS%3BCE%3BBR%3BOTH%3BCO%3BBR&utm_content=Biomarkers_B&utm_term=%2Bbiomarkers&gclid=CjwKCAjwkvWKBhB4EiwA-GHjFiHVo8gRqGjzLuibGFEUxhCf9vApcQ5r0hnYaRf44nQoWRIUnBbaBRoCtDsQAvD_BwE&gclsrc=aw.ds
Biomarker Testing Services	https://www.bioagilytix.com/services/assay-focus/biomarkers/
Biomarkers and Qualification – Food and Drug Administration	https://www.fda.gov/drugs/biomarker-qualification-program/about-biomarkers-and-qualification
Biomarkers: New Tools of Modern Medicine – Elsevier	https://www.elsevier.com/about/press-releases/archive/research-and-journals/biomarkers-new-tools-of-modern-medicine
Biosystems Acro	https://www.acrobiosystems.com/?gclid=CjwKCAjwkvWKBhB4EiwA-GHjFpkTPDe8N7wpZYSd0SNOHQhXL1Vf_kNfGGP673_Fajrz-_LFC7qAcRoCtHMQAvD_BwE
Bruker	https://www.bruker.com/en/products-and-solutions/mass-spectrometry/maldi-tof.html
Cambridge Epigenetix	https://cambridge-epigenetix.com/
COnsortium of METabolomics Studies (COMETS)	https://epi.grants.cancer.gov/comets/#:~:text=The%20COnsortium%20of%20METabolomics%20Studies,perform%20metabolomic%20profiling%20of%20individuals
Creative BIOMART Biomarkers	https://biomarker.creativebiomart.net
Electrochemiluminescent Assay – Meso Scale Discovery Platform	https://www.mesoscale.com/en/technical_resources/our_technology/ecl
Epigenetics – Promega	https://www.promega.co.uk/resources/guides/nucleic-acid-analysis/introduction-to-epigenetics/

(continued)

Table 5 (continued)

Organization or company name	Web address
Eurofins Biotherapeutics Discovery Services	https://www.eurofinsdiscoveryservices.com/services/biotherapeutics-discovery-services/
Hybrid LBA/LC-MS – Bioanalysis Zone	https://www.bioanalysis-zone.com/spotlights/hybrid-lba-lc-ms/
Illumina	https://www.illumina.com
Koneksa Digital Biomarker Platform	https://www.koneksahealth.com/platform/
Medical device Network	https://www.medicaldevice-network.com/features/biomarker-technology/
Metabolon	https://www.metabolon.com/
Op-T LLC	http://op-t.com/
Perkin Elmer	https://www.perkinelmer.com
Proximity Extension Assay – OLINK Proteomics	https://www.olink.com/data-you-can-trust/technology/
Sciex	https://sciex.com/applications/life-science-research
Thermo Fisher	https://www.thermofisher.com

This table lists technologies or platforms relevant to biomarkers or diabetes. Please note, occasionally the location of the websites or web address changes

Table 6 Other resources of interest or relevance for health care professionals or patients related to biomarkers or diabetes

Name of resource or organization	Web address
American Academy of Ophthalmology: Material on Retinopathy	https://www.aao.org/preferred-practice-pattern/diabetic-retinopathy-ppp
BEST (Biomarkers, EndpointS, and other Tools) Resource	https://www.fdanews.com/ext/resources/files/2020/11-24-20-BEST.pdf?1606261388
BioAgilytix	https://www.bioagilytix.com/
Biologic Medicine Information Center (BMICC): Urinary Protein Biomarker Database	http://upbd.bmicc.cn/biomarker/web/indexdb
Biomarker-Menu – BioAgilytix	https://www.bioagilytix.com/biomarker-menu/
Biomarkers – National Institute of Neurological Disorders and Stroke	https://www.ninds.nih.gov/Current-Research/Focus-Tools-Topics/Biomarkers
Biomarkers Consortium Resources	https://fnih.org/what-we-do/biomarkers-consortium/about/resources
Biomarkers Database – Charles River	https://wwwapps.criver.com/BiomarkersDB/
Biomarkers Definition Working Group	https://doi.org/10.1067/mcp.2001.113989
Biomarkers in disease areas – Roche	https://www.roche.com/partnering/diagnostics-areas-of-interest/new-biomarker-testing.htm
Biomarkers, Genetics and Epigenetics – Understanding Society	https://www.understandingsociety.ac.uk/topic/biomarkers-genetics-and-epigenetics
BioTech Pharma Summit	https://www.biotechpharmasummit.com
Diabetes – World Health Organization (WHO)	https://www.who.int/health-topics/diabetes#tab=tab_1
Diabetes Fact Sheets – World Health Organization	https://www.who.int/news-room/fact-sheets/detail/diabetes
Diabetes UK: Material on Living with Diabetes	https://www.diabetes.org.uk/guide-to-diabetes
Diabetes UK: Material on Local Support Groups	https://www.diabetes.org.uk/how_we_help/local_support_groups
Diabetic Retinopathy Data and Statistics – National Eye Institute (NEI)	https://www.nei.nih.gov/learn-about-eye-health/outreach-campaigns-and-resources/eye-health-data-and-statistics/diabetic-retinopathy-data-and-statistics
Division of Diabetes Translation – Centers for Disease Control and Prevention (CDC)	https://www.cdc.gov/diabetes/index.html
Euretos	https://www.euretos.com
European Federation of Pharmaceutical Industries and Associations: Working with Patient Groups	https://www.efpia.eu/relationships-code/patient-organisations/
FDA-NIH Biomarker Working Group	https://www.ncbi.nlm.nih.gov/books/NBK338449/
Genomeweb	https://www.genomeweb.com/biomarker-discovery-validation
Global Biomarker Standardization Consortium – Alzheimer's Association	https://www.alz.org/research/for_researchers/partnerships/gbsc
Global Online Biomarker Platform (GOBIOM)	https://gobiomdbplus.com

(continued)

Table 6 (continued)

Name of resource or organization	Web address
KNApSAcK human biomarker database	http://www.knapsackfamily.com/Biomarker/top.php
National Institute of Neurological Disorders and Stroke: Patient Organizations	https://www.ninds.nih.gov/Disorders/Support-Resources/Patient-Organizations
NHS: Material on Diabetes and Finding Help and Support	https://www.nhs.uk/conditions/type-2-diabetes/finding-help-and-support/
The Patients Association	https://www.patients-association.org.uk/
Urinary Protein Biomarker Database (UPBD) – BMICC	http://upbd.bmicc.cn/biomarker/web/indexdb
Vision Loss and Diabetes, American Diabetes Association	https://www.diabetes.org/diabetes/eye-health/understand-eye-conditions
WHO International Programme on Chemical Safety Biomarkers and Risk Assessment: Concepts and Principles	https://apps.who.int/iris/bitstream/handle/10665/39037/9241571551-eng.pdf
WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation	https://inchem.org/documents/ehc/ehc/ehc222.htm

This table lists other resources of interest or relevance to biomarkers or diabetes. Please note, occasionally the location of the websites or web address changes

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Correction to: Assessing Insulin Sensitivity in People with Type 1 Diabetes Without Euglycemic-Hyperinsulinemic Clamps

Andrzej S. Januszewski and Alicia J. Jenkins

Correction to: Assessing Insulin Sensitivity in People with Type 1 Diabetes Without Euglycemic-Hyperinsulinemic Clamps in Vinood B. Patel and Victor R. Preedy (eds.) *Biomarkers in Diabetes*, Biomarkers in Disease: Methods, Discoveries and Applications,

https://doi.org/10.1007/978-3-031-08014-2_18

Due to an oversight on the part of Springer, some formulas of this chapter were initially published with errors. The correct presentation is given here.

$$elnIS = 4.108 - 0.013 * WC - 1.058 * TDD - 0.313 * TG - 0.008 * DBP \quad (33)$$

$$elnIS = 4.108 - 0.013 * WC - 1.058 * TDD - 0.0035 * TG - 0.008 * DBP \quad (34)$$

$$elnIS = 7.472 - 0.013 * WC - 0.25 * HbA1c - 0.357 * G_0 + 0.019 * Adiponectin - 0.287 * TG - 0.006 * DBP \quad (35)$$

$$eGDR = -31.714 + 0.142 * Age + 2.399 * \mathrm{HDL} - \mathrm{C} + 8.613 * 1neGFR - 2.051 * VAI - 0.12 * PP \quad (42)$$

The updated original version for this chapter can be found at https://doi.org/10.1007/978-3-031-08014-2_18

V. B. Patel, V. R. Preedy (eds.), *Biomarkers in Diabetes*, Biomarkers in Disease: Methods, Discoveries and Applications,
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Index

A
Acetylcholine, 1064
Acromegaly comorbidities
  cardiovascular disease, 952, 953
  growth hormone, 956
  metabolic complications, 951, 952
Action to Control Cardiovascular Risk in Diabetes (ACCORD) Eye Study, 970
Acute myocardial infarction (AMI), 164
Acute phase reactants, 1058
Acylcarnitines, 30, 32, 119
Adenine phosphoribosyltransferase (APRT), 131
Adenosine, 134, 147
Adenosine deaminase (ADA), 146, 147
  adenosine level, 137
  inosine, 134
  isozyme activities, 138
  modulators, 137
  serum level, 137
Adenosine triphosphate (ATP), 1093
Adhesion molecules, 869, 871–873, 875
Adipocytes in brown adipose tissue, 381
Adipokines, 47, 72, 155, 222, 337, 380, 761
  biomarkers, 203
  classification, 382, 383
Adipo-myokine, 45
Adiponectin, 221, 1095, 1096, 1124
Adipose, 561
Adipose tissue, 25, 71, 72, 155, 222, 287, 301, 302, 304, 305
  pathophysiology of gestational diabetes mellitus, 380, 381
Adropin, 222
Adult stem cells
  embryonic and somatic, 561
  EPCs (*see* Endothelial progenitor cells (EPCs))
  exercise and lifestyle changes, 561
  MSCs, 566, 567
  physical exercise, 561
Advanced glycation end products (AGEs), 185, 517, 532, 658, 825, 894, 902, 917, 966, 1093
  accumulation, 173
  circulating levels, 175
  in diabetes, 174
    arterial alterations, 180
    cancer, 182, 183
    cardiomyopathy, 180
    DR, 181, 182
    effects, 177
    neuropathy, 178–181
    oxidative stress, 183, 184
  disease complications, 185
  effects, 176, 177
  importance, 185
  macrovascular disease, 184
  measuring methods, 173, 177
  monoclonal antibodies, 173
  non-enzymatic reaction, 173, 174
  origin, 174, 175
  pentosidine, 174
  pro-inflammatory cytokines, 173
  pyrraline, 174
  RAGEs, 173, 175, 176
Advanced glycosylation end products, 875–877
Aerobic, 30, 33, 47
Age-related macular degeneration (AMD), 333, 983
Akkermansia muciniphila, 926, 931
AKT kinase, 489
Albuminuria, 414–417, 471, 474, 480
Aldose reductase (AR), 1094
Aliphatic γ-diketones, 1001

V. B. Patel, V. R. Preedy (eds.), *Biomarkers in Diabetes*, Biomarkers in Disease: Methods, Discoveries and Applications,
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Alkyl formylglycosyl pyrrole (AFGP, **30**), 1007
All-cause mortality, 810, 814, 816, 818
Allodynia, 1106
Alloxan and STZ, 1117
Alloxan induced DM, 1116
Alloxan induced mild diabetic (MD), 1121
Allozymes, 440
Alpha-1-microglobulin, 900
Alpha-linolenic acid (ALA), 452
Altered SUA levels, 243
Alzheimer's disease (AD), 71, 184, 322, 325
Ambulatory blood pressure monitoring (ABPM), 707
American Diabetes Association (ADA), 377, 521, 762, 1027
Amino acid(s) (AA), 85, 242, 282, 1039–1043
  BCAA (*see* Branched-chain amino acids (BCAA))
  in diabetes, 91, 92, 113–118
  in insulin resistance, 91–93, 113–118
  metabolism, 301
  sequences, 437
  T2DM, 93
γ-Aminobutyric acid (GABA) receptors, 285
AminoIndex LifeStyle diseases (AILS), 117
β-Aminoisobutyric acid (BAIBA), 119
Ammonium sulphate salt, 799
Amylin, 1060
  accumulation, 1060
  induced inflammatory response, 1060
Amyloid-beta
  high blood glucose, 1060
  plaque formation, 1060
Amyloid deposition, 290
Ancestry-informative markers (AIMs), 674, 675, 677
Anemia, 5
Angiogenesis, 659, 968, 977, 982
AngiomiR, 659
Angiopoietin-like 4 (ANGPTL4), 222
Angiopoietin-like protein 8 (ANGPTL8), 381
Angiopoietins 1 to 4 (Ang 1-4), 979
Antidyslipidemic properties of SC, 1123
Anti-inflammatory
  acute effects, exercise, 45, 46
  chronic effects, exercise, 47
  exercise interventions, 47, 48, 54
Antimicrobial peptides (AMPs), 929
Antimicrobial proteins, 929
Aortic pulse wave velocity, 856–859
APOE4-homozygous patients with Alzheimer disease, 1006
Apolipoprotein AV (ApoAV), 402
Apolipoprotein C3 (APOC3), 401, 402
Apolipoprotein C3-transgenic (ApoC3-tg), 400
Apolipoprotein E, 693
Aqueous humor (AH), 972
Arachidonic acid (AA) in the cell membranes, 454
Area under the curve (AUC), 338
Aromatic amino acids (AAAs), 87
Aryl hydrocarbon receptor (AHR), 929
Association study, 624
Astrocytes, 1096
Asymmetric dimethyl-arginine (ADMA), 158, 563, 870, 878
Atherogenic, 1106
Atheromatosis process, 823
Atherosclerosis, 443
Atherosclerosis Risk in Communities (ARIC) study, 810
Atrial fibrillation, 817
Augmentation index, 857
Autoimmune Disease, 462
Automated electronic (oscillometric) devices, 736
Avignon index, 354

B

Bacterial translocation
  bacterial DNA, 936
  diseases/conditions, 937
  lipopolysaccharide (LPS), 935, 936
  mechanism, 934
  microbial products/fragments, 933
  trigger inflammation, 933
  type 2 diabetes mellitus, 938–940
Bacteroides caccae, 931
Batokines, 380, 381, 384
B-Cell Stimulating Factor, 978
BCKA dehydrogenase (BCKD), 301
Belfiore index, 354
Bennett index, 356
Betaproteobacteria, 927
Betatrophin, 381
  in gestational diabetes mellitus, 385–387
  on human beta-cell proliferation, 384
  lipoprotein lipase (LPL) activity in vitro, 385
  physiological (postprandial state) and pathological circumstances, 387
Betatrophin mRNA levels in the liver of the *ob/ob* and *db/db* mouse models, 388
Biochemically stable epigenetic markers, 545
Biochemical markers, 763, 764

Biomarker(s), 146, 156, 157, 164, 240, 331, 471, 477, 479–481, 577, 1035
applications, 816
cardiovascular causes, 810
C-reactive protein, 333, 334, 337, 338
diabetes, 812
diabetes mellitus (DM), 810
ELISA, 339
human saliva, 335
multiple sclerosis, 578–579
multiplex analysis, 340
myocardial action potential, 810, 811
pathologic Q waves, 815
QT interval, 811, 812
REGARDS study, 815
type 1 diabetes mellitus, 577–578
type 2-diabetes, 331, 332
Biomarkers of diabetes mellitus, 1142
books on, 1147
journals related to, 1142–1146
professional societies, 1145
regulatory bodies dealing with, 1144
techniques and platforms related to, 1142–1150
Biosensor, 341
Biosynthesis, 282–284
Bisulfite conversion, 600, 602, 607, 609
Bisulfite conversion-based methods, 600
Blood-brain barrier (BBB), 285
Blood glucose, 616–619, 621–626
levels, 241
Blood glucose monitoring, 1023
protocol, 1026
technique, 1022
Blood pressure (BP), 730, 733, 734, 970
Blood pressure variability (BPV), 708
index, 709
types, 708, 710
Blood retinal barrier (BRB), 965, 983
Blood vessels, 561
Body fluid status, 416
Body mass index (BMI), 24, 133, 200, 378
Bone marrow, 561
Brain, 1056–1059
Brain derived neurotrophic factor (BDNF), 1096
cardiovascular disorders, 487
central and peripheral nervous systems, 487
central nervous system disturbances, 503
central sensitization, 506
deficiency, 506
diabetes mellitus, 505, 506
diabetic polyneuropathy, 499–502
gluconeogenesis, 506
G-protein coupled receptors, 505
growth factors, 487
human populations, 493
inflammation, 494, 495
insulin resistance, 506
leptin, 506
microangiopathic complications, 487
mitogenic effect, 504
nervous system, 487, 506
neuropathic pain, 503, 506
neuroplasticity, 506
neuroplasticticity effects, 487
neuroprotective effects, 504
neurotransmitter modalities, 487
neurotrophins, 502
p75, 506
pancreatic beta cells, 506
pathological processes, 489
Ras membrane protein, 488
streptozocin-induced diabetes, 506
type 2 diabetes mellitus, 490, 492
Brain related functions, 462
Branched-chain amino acids (BCAA), 242, 301, 305, 306
accumulation, 122
acylcarnitines, 119
association, 120
circulation, 113, 117
concentrations, 91, 113
dysmetabolism, 90, 122
enzymatic activity, 87
FAA profiles, 113
health-promoting effects, 121
hepatic metabolic disorders, 89
metabolic pathway, 89
metabolism, 90, 91
mitochondrial oxidation, 90
mTORC1 activation, 89, 121
nutritional signals, 89
positive associations, 115
supplementation, 113, 121
vs. T2DM development, 114, 121
Branched-chain α-keto acids (BCKAs), 301
Breast cancer, 75
Brown adipocyte, 305

C

Calciferol (Vitamin D), 228
Calcium (Ca) dependent hydrolase, 438
cAMP-response element binding protein (CREB), 505
Canadian Diabetes Association guideline, 1027

Canagliflozin (CG), 565
Cancer, 182, 183
  CRCs, 75
  invasive/non-invasive, 75
  signs, 75
  symptoms, 75
Carbohydrate metabolism, 242
Carbohydrates, 1045
Cardiac fibrosis, 828
Cardiac magnetic resonance imaging, 843
Cardiac metabolism, 827
Cardiac myocytes, 810
Cardiometabolic dysfunctions, 23
Cardiomyopathies, 826
  ischemic versus non-ischemic, 834
  non-invasive imaging, 834
Cardiovascular and cerebrovascular disorders, 461
Cardiovascular disease (CVD), 71, 73, 134, 146, 313, 320, 321, 333, 341, 397, 402, 517, 560, 565, 690–692, 952, 953
Cardiovascular magnetic resonance imaging (CMR), 824, 843
Cardiovascular risk, 869, 871–873, 879
Carotid body(ies) (CBs)
  adenosine, 755
  applications, 768
  arterial blood gases, 753
  autonomic nervous system, 753
  blood flow, 753
  CBmeter prototype, 765
  chemoreceptor organs, 753
  chemoreflexes, 754
  chemosensitivity, 759, 768
  chemotransduction, 754
  diabetes, 767
  glomerulus, 755
  hyperreflexia, 768
  hyperreflexivity, 760, 761
  hypertonicity, 768
  insulin, 757
  leptin, 757, 758
  metabolic diseases, 753, 755–757
  nervous system, 753
  pathology, 767
  pathophysiological mechanisms, 753
  peripheral chemoreceptors, 759
  plethysmographic recordings, 759
  sensory nerve, 753
  sustentacular cells, 755
  techniques, 758
  type II cells, 755
Carotid-femora, 859
Carotid sinus nerve (CSN), 753, 768
Caveolin-1 (CAV-1), 1105
CBmeal, 768
CBmeter, 768, 770
CBview, 769
C-C motif ligand 2 (CCL2), 1100
CD163 receptor, 687, 689
CD34+ve progenitor cells, 565
Cederholm and Wibell Index, 353
Cell culture, 478
Cell-free DNA (cfDNA), 598, 609
β cell(s), 609
  function, 950, 953
  pancreas, 286
Cell-to-cell communication, 974
Cellular process, 471
Cellular receptors for AGE (RAGE) products, 997
Central nervous system (CNS), 487, 1057
Central obesity, 242
Central-peripheral distal axonopathy (CPDA), 995, 996
Centre of Disease and Prevention (US), 792
Ceramide, 28, 90
Cerebral microcirculation, 293
Chemokine(s), 137, 337, 589, 929
  receptors, 589
Chemokine ligand 2 (CCL2), 978
Chlorpyrifos, 438
Choline acetyltransferase and cholinesterase, 1064
Cholinergic dysfunction, 1064, 1066
Choriocapillaris flow impairment, 783
Chromium (Cr), 288
Chromophore generation, 1004
Chronic diseases, 196
Chronic exposure to diabetic environment, 265, 266
Chronic hyperglycemia, 88, 442, 893, 1059, 1063
Chronic inflammation, 528
Chronic insulin resistance, 379
Chronic kidney disease (CKD), 178, 323, 523, 524
Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, 362
Chronic sustained hyperglycemia in diabetes mellitus, 658
Chronic wounds, 317
Circulating cell-free DNA (cfDNA), 545

Circulating miRNAs, 651, 657, 658
and diabetes, 659
and DR, 653, 655, 659, 660
Circulatory biomarkers, 869–877
Classic lipid biomarkers, 33
Clinical grading of DR, 779
Clinically isolated syndrome (CIS), 578
Clinically used biomarkers in Diabetes mellitus, 1116, 1117
Co enzyme A (CoA), 302
Cognitive dysfunction, 294, 295
Cognitive dysfunction and impairment, 1063–1064
Cognitive function, 282, 290
domains, 291
factors, 292, 293
hyperhomocysteinemia, 293
screening tools, 291, 292
Colorectal cancers (CRCs), 75
Complement system (CS), 981
Complete Freund's adjuvant (CFA), 587
Complications of diabetes, 325
Continuous glucose monitoring (CGM), 526, 1024, 1025
Corneal nerve degeneration, 421
Coronary artery calcification (CAC), 73
Coronary Artery Calcification in Type 1 Diabetes (CACTI) cohort, 359
Coronary artery disease (CAD), 443, 560, 562
Coronary atheromatosis, 826
Coronary heart disease, 333
Cortical atrophy, 1057
Cost-effective, 335
COVID-19 pandemic, 568, 718
C peptide, 1121
CpG methylation, 599
c-reactive protein (CRP), 73, 332, 333, 494
Ctokines, 78
CXCR4 receptor, 565
Cyclic adenosine monophosphate, 795
Cysteine, 282
Cytokines, 42, 46, 55, 337, 974, 977–979

D

Dabelea formula, 358
Damage-associated molecular patterns (DAMPs), 265
Day-by-day HBPV, 710
albuminuria, 714
eGFR, 716–718
target organ damage/CVD, 710, 711
Dejour test, 759, 764
Dendritic cells (DCs), 929
De novo lipogenesis (DNL), 25
Desmosomes and adhesive junctional proteins, 928
Diabesity cardiac function, 827
Diabetes, 74, 75, 88, 241, 331, 524, 560, 671, 812, 925
adulthood, 731
applications, 742
atherosclerotic cardiovascular disease, 731
biomarkers, 88, 89
blood pressure measurement, 733, 734
brain, 1056–1059
cardiovascular risk factor, 730
characteristics, 1055
chronic disease, 69
complications, 1056
death and cardiovascular outcomes, 730
economic and health problems, 69
hypertension, 730
macrovascular complications, 69
methodology, 738
microvascular complications, 69
nephropathy, 731
neuroinflammation, 1057–1059
prevalence, 731
progression of, 1063
"tracking" phenomenon, 730
types, 1055
Diabetes-associated complications, 532
Diabetes Control and Complications Trial (DCCT), 524, 970
Diabetes-induced cognitive dysfunction, 1057
Diabetes-induced neuroinflammation, 1059–1063
cognitive decline, 1056, 1065
Diabetes mellitus (DM), 87, 220, 289, 313, 616–618, 620, 623, 625, 810, 893, 1065
blood-brain barrier disruption, 1058
burden of diabetes, 949
cell function, 949
β-cell mass, 949
complications, 949
C-peptide, 950
glycation, 518–520
growth hormone (GH), 951
hyperglycemia, 518, 949
hyperinsulinemic-euglycemic clamp technique, 950
inflammation, 951
insulin resistance, 950
insulin secretion, 950
metabolic stress, 951

Diabetes mellitus (DM) (*cont.*)
pathogenesis of, 1055
pathophysiology, 1113
prevalence, 993
Diabetic cardiomyopathy, 825, 842
Diabetic encephalopathy, 1056, 1059
Diabetic eyes without clinical retinopathy, 780
Diabetic foot syndrome (DFS), 498
Diabetic heart disease, 825
Diabetic keratopathy, 421, 422
Diabetic ketoacidosis (DKA), 577
Diabetic kidney disease (DKD), 315–317, 568, 893, 894, 904
classified by albuminuria and eGFR, 713
progression, 713
risk factors for end-stage renal disease, 711
without albuminuria, 711
Diabetic macular edema (DME), 415, 783
Diabetic microvascular complications, 251
Diabetic nephropathy (DN), 252, 289, 317, 470, 472, 477, 480, 553, 554, 691, 692, 800, 816, 1092, 1093, 1107
concentrations, 138
conventional biomarker, 895
diabetic subjects, 138
genomics, 904
glomerular biomarkers, 897
glomerular damage biomarkers, 896–898
inflammatory biomarkers, 900, 901
metabolic profile, 139
metabolomic profiling, 904
metabolomics studies, 138
miRNAs, 903
NADPH oxidase, 903
oxidative stress, 901, 902
pathogenesis of, 893
proteomics, 903
stages of, 894
tubular biomarkers, 899
tubular damage biomarkers, 898–900
Diabetic nephropathy treatment on diabetic retinopathy and diabetic macular edema, 417, 418
Diabetic peripheral neuropathy (DPN), 317
Diabetic polyneuropathy, 487, 490, 498, 499, 504, 1009
Diabetic retinopathy (DR), 138, 181, 182, 315, 413, 551–553, 652, 670, 677, 779, 816, 912, 917
clinical grading, 968
clinical management, 969–971
miR-1281, 663
pathogenesis, 966–968
Diabetic Retinopathy Severity Scale (DRSS), 969
Diacylglycerol (DAG), 26, 90, 91
Dialysis, 526, 528–530
Diastolic function, 836, 841, 843
Diazinon, 438
Dichlorofluorescein, 904
Diet and Polyunsaturated fatty acids, 456, 457
Dietary intake, 793
Digital PCR, 600, 604
γ-Diketone, 1005
γ-Diketone-derived pyrroles, 1003, 1004
Dipeptidyl peptidase-4 (DPP4), 202, 203, 205, 222, 223
Dipeptidyl peptidase-4 (DPP-4) inhibitors, 203, 420, 563
Distal axonopathy, 995
Distal symmetrical polyneuropathy (DSPN), 497, 994, 995
clinical biomarkers, 998
etiology, 996
pathogenesis, 996
Diurnal variation, 1025
DNA methylation, 544
DNA methyltransferases, 599
DN pathophysiology, 478
Docosahexaenoic acid, 455
Downstream regulatory element antagonist modulator (DREAM), 1096
Drug discovery, 982, 983
Duca formula, 359
Dysfunction in the glomerular filtration, 263
Dysglycemia, 524
Dyslipidemia, 23, 24, 129, 146, 242, 249

E

ɣ-amino butyric acid B (GABAB) receptor, 1102
Early Treatment Diabetic Retinopathy Study (ETDRS), 969
Echocardiography, 844
eGFR and serum uric acid levels, 252, 253
eGFR decline in patients with lower eGFR, 712
Eicosanoids, 455
Eicosapentaenoic acid, 454
Electrocardiogram (ECG), 810, 811
Electrochemiluminescent (ECL) assay, 974, 975
Electrospray ionization (ESI), 794
Elongation and desaturation of Omega-3 and Omega-6 fatty acids, 454
Endocan, 877

End of stage kidney disease (ESKD), 524
Endothelial dysfunction, 249, 850
Endothelial function
  aortic pulse wave velocity, 856–859
  applications to diabtes, 860
  characteristics of technique for measurement of, 851
  flow-mediated dilatation (FMD), 854–856
  laser doppler flowmetry, 859–860
  non-circulatory measures of, 851–860
  peripheral artery tonometry, 856
  skin autofluorescence, 860
  venous occlusion plethysmography, 852–854
Endothelial function, in type 2 diabetes
  adhesion molecules and chemokines, 872–873
  advanced glycosylation end products, 875–877
  applications to diabetes, 878–879
  circulatory biomarkers, 869–877
  endocan, 877
  endothelial microparticles, 874–875
  endothelial progenitor cells, 873–874
  external counterpulsation therapy, 877–878
  high sensitivity C-reactive protein, 871–872
  nitric oxide metabolites and asymmetric dimethylarginine, 869–871
Endothelial microparticles (EMP), 874–875
Endothelial progenitor cells (EPCs), 568, 569, 873–874
  age, 562
  effect of HIV medication regimens, 565–566
  effect of novel diabetes medications, 563–565
  metabolic disorders, 562
Endothelin-1, 175
Endotoxin core antibodies (EndoCAb), 937
End-stage renal diseases (ESRD), 470
Engulfment and cell motility protein (ELMO-1), 471–481
Enterobacterales, 932
Enterococci, 932
Enzyme dysfunction, 175
Enzyme-linked immunosorbent assay (ELISA), 173, 177, 339, 340, 387, 974
Epigenetic biomarkers for T2D-associated complications, 550
Epigenetics
  advancing technologies, 603–605
  applications, 607
  bisulfite conversion, 602
  β-cell, 598
  β-cell death, 601, 603, 604, 608
  cell-free DNA (cfDNA), 599, 600
  DNA methylation detection, 600, 601
  limitations, 606, 607
  mechanisms of cell death, 598
  modifications, 598, 599
  T1D, 605, 606
Epithelial cell layer, 929
Epithelial to mesenchymal transition (EMT), 76
Equations, 355, 357, 364
Erythrocyte-related HbA1c variants
  diabetes screening, 12
  G6PD
    G6PD deficiency, 16
    rs1050828, 12, 14, 15
    rs76723693, 12
  HBB, 9
    rs334, 13
    rs334, 14, 15
    rs334, 9
  HBM, 11
Erythropoietin (EPO), 414, 978
E-selectin, 175
Estimated glomerular filtration rate (eGFR), 413, 619, 620, 626
Ethnicity, 670–674, 676, 677
Eugenia Jambolana (EJ), 1113
Euglycaemic hyperinsulinaemic clamp, 351
  glucose disposal rate, 352
  insulin concentration, 352
  insulin sensitivity, 353
  insulin sensitivity indexes, 353
  metabolic clearance rate of glucose, 352
  M/I ratio, 352
EURODIAB Insulin-Dependent Diabetes Mellitus Complications Study (EURODIAB IDDM), 812
European Society for the Study of Diabetes (EASD), 762
Evolutionary norms of the n-6 to n-3 PUFA, 457
Excitotoxicity, 285
Exercise, 563, 569
Exosomes, 569
Experimental autoimmune encephalomyelitis (EAE) model, 587
Experimental DM models
  type 1 DM, 1116–1118
  type 2 DM, 1119
External counterpulsation (ECP), 877–878
Extracellular glutathione peroxidase (eGPx), 316

Extracellular matrix (ECM), 177, 471, 474, 480, 481
Extracellular protein kinase (ERK), 955
Extracellular signal-regulated kinases (ERKs), 285
Extracellular volume, 832

F
FA desaturases, 454
Faecalibacterium prausnitzii, 931
Fasting blood glucose (FBG), 794, 950, 1119, 1120
Fat metabolism, 390
Fatty acid(s) (FAs), 451
  in cancer development, 461
  oxidation, 305
  supplementation, 461
  uptake, 302
Federal Drug Association (FDA), 526
Fetuin-A, 206, 223, 224
Fetuin-B, 207, 223, 224
Fibrinogen, 177
Fibroblast growth factor 21 (FGF21), 224
Fibronectin, 474, 896, 898
Fibrosis-quantitation, 829, 830
Finger plethysmography, 856
Finnish Diabetes Risk Score (FINDRISC), 762
Finnish National Diabetes Program, 762
Flow-mediated dilatation (FMD), 854–856
Forkhead box protein O1 (FOXO1), 638, 644
Formulation of Ehrlich's reagent, 1007
FoxP3 expression, 585
Framingham Heart Study (FHS), 113
Free amino acids (FAAs), 85, 242
Free fatty acids (FFAs), 24, 90, 459, 632
Frizzled transmembrane receptors (FZD), 633
Fructosamine, 519, 522–524

G
Gadolinium, 843
  contrast, 829, 831, 832, 844
Gadolinium hyperenhancement (LGE) technique, 829
Gallic acid, 1128
  and ellagic acid binding modes, 1129
Gamma-amino butyric acid (GABA) receptors, 1102, 1103
Gas chromatography, 173
Gas chromatography coupled with mass spectrometry (GC-MS), 177
Gastrointestinal tract (GIT), 925, 941
Gemigliptin, 223
Gene bodies and enhancer markers, 546
Genetic(s), 671, 672, 674, 676, 677
  models, 1119
  polymorphisms, 401, 402, 405
  variant, 16, 471, 472, 478, 480
Genetic risk score (GRS), 16
Genome-wide association study (GWAS), 5, 16, 400, 405, 471, 577, 673, 904
Genome-wide profiling technologies for 5-hydroxymethylation, 547
Genomic ancestry
  ancestry-informative markers (AIMs), 674
  applications, 676
  clinical practice, 671
  diabetic retinopathy (DR), 670
  ethnical groups, 674
  ethnicity, 672
  inflammation, 671
  insertion-deletions (Indels), 673
  methodologies, 671, 672
  multi-ethnic cohorts, 676
  multiple genetic markers, 671
  oxidative stress, 671
  short tandem repeats (STRs), 673
  single nucleotide polymorphisms (SNPs), 673
  sociodemographic factors, 674
Gestational diabetes, 606
Gestational diabetes mellitus (GDM), 205, 1034, 1078–1079
  adipogenesis, 640
  adipose tissue expansion, 640
  applications, 641, 642
  applications of metabolomics, 1039
  biological pathways, 636
  biomarker, 640, 641
  consequences, 1036
  cut-off values, 1023
  defined, 1022
  diagnosis, 378, 1037, 1038
  epidemiology, 1035
  etiology, 1035
  glucose homeostasis, 631
  hepatocytes, 637
  inflammation, 636
  inflammatory state, 635
  insulin resistance, 639
  insulin target tissue, 636, 639
  metabolic heterogeneity, 1038, 1039
  metabolic stress condition, 632
  obesity, 638
  oxidative stress, 635, 636

pancreatic ß-cells, 637
physiology, 379, 380
pregnancy, 632
prevalence, 378
prevention/prediction, 1039, 1040
risk factors, 378
screening, 378, 1037, 1038
treatment, 378, 632, 633
trophoblast cells, 635
Wnt ligands, 635
Wnt signaling pathway, 635
GH-releasing hormone (GHRH), 951, 959
Glaucoma in Diabetes, 422
Glial cells, 978, 1096, 1097
Glial fibrillary acidic protein (GFAP), 1096
Globular adiponectin therapy, 221
Glomerular base membrane (GBM), 474
Glomerular filtrate, 625
Glomerular filtration barrier, 474
Glomerular filtration rate (GFR), 543, 893
Glomerular hyperfiltration, 414
Glomerular hypertrophy, 894
Glomerulosclerosis, 474
Glomerulo-tubular, 904
Glucagon-like peptide-1 (GLP-1), 223, 636, 926, 1124
agonists, 418
receptor, 205
signaling, 1080
Gluconeogenesis, 242, 625
Glucoronate, 904
Glucose, 174, 519, 1056
homeostasis, 617, 631, 1077, 1078, 1080–1081
induced cardiac inflammation, 1126
metabolism, 404, 769, 1057
Glucose challenge tests (GCT), 1038
Glucose disposal rate (GDR)
during euglycaemic hyperinsulinaemic clamps, 362
type 1 diabetes, 357–361
Glucose-regulated protein (GRP78), 159
Glucose Regulation during Healthy Pregnancy, 379
Glucose synthase kinase-3 (GSK-3)
phosphorylation, 1063
upregulation of, 1063
Glucose transporter 4 (GLUT-4), 28, 209, 439, 444
Glucose transporter carrier proteins (GLUT), 797
Glucosuria, 616–618, 620–621, 625
Glutamic acid, 114
γ-Glutamyltransferase (GGT), 141
Glutathione (GSH), 1094
Glutathione peroxidase (GPX), 1076, 1095
Glycated albumin (GA), 800
applications, 531
chronic hyperglycemia, 530
conventional measures, 521, 522
enzymatic method, 530
fructosamine, 522–524
glycation products, 529
hemoglobin, 522
history, 515
laboratory measurement, 530
Maillard reaction, 516
metabolic diseases, 529
monitoring metabolic control, 520, 521
observational studies, 527
osmotic pressure, 515
pH balance, 515
process, 516–518
single polypeptide chain, 515
structure, 515, 516
treatment monitoring, 531
type 2 diabetes mellitus, 519
Glycated hemoglobin (HbA1c), 524–526, 543, 691, 763
Glycemia, 241
Glycemic control on the anti-VEGF therapy for DME, 419
Glycemic variability, 812, 813
Glycine, 113
Glycogen synthase kinase-3 beta (GSK3β), 489, 638
Glycolytic intermediate 1,3-bisphosphoglycerate, 998
Glycoprotein 130 (gp130), 955
Glycosaminoglycans (GAGs), 896
Glycosylated hemoglobin (HbA1c), 438, 444, 1121
Granulocyte colony stimulating factor (G-CSF), 505
Granulocytes, 528
Growth hormone deficiency (GHD), 956
Growth hormone secretion, 959
Gut dysbiosis, 286, 941
Gut microbiome, 568, 569
bacteria, 926
barrier's defense mechanisms, 927
biological signals, 926
defense mechanisms, 930
dietary fibers, 926
disruption/dysfunction, 932, 933
enteroendocrine L cells, 926

Gut microbiome (*cont.*)
integrity, 930, 931
layers, 928
mechanisms, 931
metabolites, 926
metagenome-wide-association-study approach, 926
multiple layers, 927, 929, 930
pathogenic microbes, 925
signaling channels, 926
Gutt index, 354

H
Haptoglobin (Hp), 902
biological functions, 685
correlations of, 686–689
structure and polymorphic variants, 684–685
variant and cardiovascular diseases, 690–692
variant and diabetic complications, 691–693
variant and immune-mediated diseases, 695–696
variant and infection, 693–694
HBP level and albuminuria, 713, 714
HBP level and eGFR, 714, 716
Heart failure (HF), 823, 842
Helper lymphocytes, 689
Hematopoietic stem and progenitor cells (HSPC), 567
Heme oxygenase-1 (HO-1), 1104, 1105
Hemodialysis, 529, 532
Hemodynamic stress, 716
Hemoglobin, 8, 16, 687, 690, 694, 696
Hemoglobin A1C/glycated haemoglobin (HbA1c)
erythrocyte-related genetic variants, 9, 11
clinical implication, 13
cumulative effect, 15
functional characterization, 7, 8
functional classification, 7
glycemic control, 4
glycemic-related genetic variants, 8, 9
G6PC2, 8
GCK, 8
GWAS, 5, 6
hematologic conditions, 5
unclassified genetic variants, 11
See also Erythrocyte-related HbA1c variants
Hemoglobinopathy(ies), 5, 532
Hemoglobulin (HbA1c), 800
Hemolytic processes, 528
Hepassocin (HPS), 199, 208
Hepatocyte growth factor (HGF), 977, 979
Hepatokines, 196, 198, 201–205, 208, 209
Hexokinase, 1128
HF with preserved ejection fraction (HFpEF), 823, 824
High-density lipoprotein (HDL), 73, 221, 332, 397, 437, 951, 1040
High-density lipoprotein cholesterol (HDLc), 24, 199
Higher insulin resistance, 363
High fat diet/sucrose/fructos, 1119
High glucose levels, 265
High-intensity interval training (HIIT), 33
High mobility group box 1 protein (HMGB1), 224, 225
High-performance liquid chromatography (HPLC), 525
High sensitivity C-reactive protein (hsCRP), 871–872
High-throughput RNA-Seq analysis, 660
5hmC, 549
5hmC-based diagnostic models in cfDNA and, 551
5hmC-Seal assay, 548
5hmC-Seal method, 548, 549
1H-NMR spectroscopy, 405
HOMA β and HOMA–IR, 1122
HOMA-IR, 389
Home blood pressure monitoring (HBPM), 718, 740, 741
Homeostasis mechanism, 793
Homeostatic model assessment (HOMA), 164, 353, 764, 1122, 1123
Homeostatic model assessment for insulin resistance (HOMA-IR), 133, 160, 202, 314, 388, 438
Homeostatic Model Assessment Test (HOMA), 950
Homocysteine, 281–284, 294
Homocysteine thiolactonase (HTLase) activity, 442
Homocysteinylation, 285
Hormone, 757
Hormone-sensitive lipase (HSL), 200
Hp1-1
biological activity, 697
biological functions, 685
and cardiovascular disease, 690
correlations, 689
diabetic nephropathy, 692
infectious diseases, 696
and neurodegenerative diseases, 693
structural variants, 685

Hp2-1
biological activity, 697
diabetes, 690
IL-6 and IL-10 production, 689
macroalbuminuria, 692
sCD163, 689
structural variants, 685
Hp2-2
biological activity, 697
diabetes, 690
macroalbuminuria, 692
sCD163, 689
structural variants, 685
HpDel, 686
Human aortic endothelial cell (HAEC), 158
Human endothelial progenitor cells (EPCs), 561
Human metabolome database, 240
Human umbilical vein endothelial cells (HUVEC) were, 661
Hydrogen peroxide (H_2O_2), 1094
Hydrophobic interaction, 799
3-Hydroxyisobutyrate (3-HIB), 118
amino acid metabolism, 300
biological variability, 300
heart disease/cardiovascular diseases, 305
hepatic fatty acid oxidation, 303, 304
inter-organ metabolic crosstalk, 302, 303
metabolic organs, 307
metabolomics profiling, 301
overweight, 300
2-Hydroxyisobutyrate, 904
3-Hydroxyisobutyryl-CoA hydrolase (HIBCH), 302
Hydroxymethylation Biomarkers, 546, 548
Hydroxymethylcytosines, 545
Hyperalgesia, 1106
Hyperaminoacidemia, 87
Hypercoagulation, 494
Hyperglycemia, 290, 518, 530, 671, 903, 931, 966, 968, 1035–1038, 1044, 1047, 1059, 1127
diabetic rats, 1060
Hyperglycmia and Adverse Pregnancy Outcome Study (HAPO), 1023
Hyperglycemia–driven neuroinflammation, 1059, 1061, 1065, 1066
Hyperglycemia-induced cognitive impairment, 1064
Hyperhomocysteinemia (HHcy), 281
amino acids, 282
applications, 294
cognitive dysfunction, 294
diabetes management, 294
diabetes mellitus, 289
homocysteine, 282–284
serum homocysteine, 294
Hyperinsulinemia, 761, 770, 827, 959
Hyperleptinemia, 770, 954
Hyperlipidemia, 143, 147, 952
Hypertension, 143, 146, 147, 518, 560, 671, 730–732
and diabetes, 707
Hypertension-associated nephrosclerosis, 711
Hypertriglyceridemia, 143, 147
Hyperuricemia, 253
Hypoglycemia, 290, 813
Hypoxanthine-guanine phosphoribosyltransferase (HGPRT), 131
Hypoxia-inducible factor (HIF), 133
Hypoxic ventilatory response (HVR), 758–760, 769

I

ICT-based multisensor home and ambulatory BP monitoring (IMS-ABPM), 718
IFNγ receptor, 580
IL-8 (CXCL8), 270
Immuno-based assays, 313
Immunoglobulin G (IgG), 896
ceruloplasmin, 896
Immunological assay using antibodies to pyrrole adducts, 1007
Impaired fasting glucose (IFG), 248, 761, 769, 949
Impaired glucose tolerance (IGT), 761, 769, 952
Inducible NO synthase (iNOS), 870
Infiltration of immune cells, 264
Inflammasome, 265
activation, 266
Inflammation, 40, 333, 399, 770
and diabetic kidney disease, 264, 265
IL-1β, 44, 45
IL-6, 43, 44
obesity, 40
and oxidative stress in experimental diabetes, 1127
TNF-α, 42, 43
type 2 diabetes mellitus, 42
Inflammation-induced processes, 265
Inflammation-related processes, 264
Inflammatory biomarkers, 1125, 1126
Inflammatory cytokines, urinary levels, 264
Inflammatory diseases, 568
Inflammatory kidney conditions, 529

Inflammatory markers, 929, 1099
Inflammatory mediators, 265
Inflammatory proteins, 1065
Inosine, 147
Inosine-5′-monophosphate dehydrogenase (IMPDH), 139, 141
Insertion-deletions (Indels), 673, 675, 677
Insulin, 601, 604, 606, 609, 610, 757
  to glucose ratio, 356
  levels, 241
  secretion, 1035, 1036, 1038, 1043, 1046, 1047
  sensitivity, 1036, 1038, 1040, 1042, 1043, 1046, 1047
  sensitivity index, 355
Insulin dependent diabetes mellitus (IDDM), *see* Type 1 diabetes
Insulin-like growth factor-1 (IGF-1), 951
Insulin-like growth factor 1 receptor (IGF1R), 551
Insulin receptor (INSR), 287
Insulin receptor substrate-1 (IRS-1), 439, 637, 644
Insulin resistance (IR), 25, 26, 29, 85, 129, 147, 156, 287, 288, 290, 294, 305, 363, 379, 436, 444, 1077, 1080–1082
  biomarkers, 197, 198
  cardiovascular risk factors, 199
  estimation based on neural-network analysis, 360
  fatty liver, 197
  liver biomarkers and functions, 202
  minerals
    calcium, 226, 227
    chromiun, 227
    magnesium, 226, 227
    selenium, 227
    zinc, 227
  obesity, 199
  visceral fat, 199–201
  vitamins
    C and E, 228
    D, K and B, 228, 229
Integrin like kinase (ILK), 474
Intercellular adhesion molecule-1, 175
Intercellular tight junctions, 928
Interferon-gamma (IFN-γ), 931, 955
Interleukin 10 (IL-10), 270, 551
Interleukin 18 (IL-18), 270, 271
Interleukin-1β (IL-1β), 635, 1100
Interleukin 1 (IL-1), 267, 268
Interleukin-6 (IL-6), 73, 134, 225, 268, 269
Interleukin (IL)-2, 955
Interleukins, 225
International Association of Diabetes and Pregnancy Study Groups (IADPSG), 632
International Diabetes Federation (IDF), 87, 518, 761, 1028
Intestinal alkaline phosphatase (IAP), 927
Intestinal epithelium, 928
Intestinal fatty acid binding protein (iFABP), 935
Intracellular adhesion molecule-1 (ICAM-1), 517, 1105
Intrahepatic lipid (IHL), 25
Intrahepatic triglyceride (IHTG), 201
Intramyocellular lipids (IMCL), 26, 29–31
Intron, 472, 478, 481
IR and insulin signalling, 1122
Irisin, 225
Iron (Fe), 288
  deficiency, 5
Iron-regulated surface determinants (IRSD), 694
Ischemic heart disease (IHD), 823
Islets of pancreas, 1126
Islet transplantation, 605–607, 610
Isoleucine, 114

J

Januszewski / Jenkins formula, 361
Japan Ambulatory Blood Pressure Monitoring Prospective (JAMP) study, 710
Japan Diabetes Clinical Data Management study (JDDM), 712
Japan Morning Surge-Home Blood Pressure (J-HOP) study, 707
J-HOP study, 713

K

Kallikrein-related peptidase, 164
Kidney damage
  associated with diabetes, 264, 270, 271
  in diabetes, 271
Kidney transplantation, 421, 422
Kinin-kallikrein system (KKS), 979, 981
Korea National Health Insurance Service Database, 423

L

Laminin, 896, 898
Laser doppler flowmetry, 859–860
Late gadolinium enhancement (LGE) technique, 832

Latent autoimmune diabetes in adults (LADA), 578
Left ventricle (LV) hypertrophy, 823
Leptin, 757, 758
Leptin receptor (LRb), 955
Leptin-to-adiponectin ratio (LAR), 221
Leucine, 114
Leukocyte cell-derived chemotaxin 2 (LECT2), 197, 207, 208
Lifestyle changes and specific prevention programs, 241
Lifestyle modification (diet and exercise), 378
Limbus, 984
Limulus Amoebocyte Lysate (LAL) assay, 935
Linoleic acid, 453
α-Linolenic acid, 453
Lipasin, 381
Lipid intermediates, 23, 26, 42
Lipidomic biomarkers of diabetes, 242
Lipidomics, 402–404, 451
Lipid(s), 356
  accumulation, 24
  cell membrane structure, 23
  energy production, 23
  free-fatty acids, 24, 28
  insulin resistance, 26
  intermediaries, 26
  mechanism, 25
  nonalcoholic fatty liver disease, 25
  obesity, 24
  profile, 1043, 1044, 1123
  storage, 302, 304
Lipocalin-type prostaglandin D synthase (L-PGDS), 896
Lipolysis, 952
Lipopolysaccharide (LPS), 927, 935, 936
Lipoprotein lipase (LPL), 32
Lipoproteins, 397, 398
Lipoteichoic acid (LTA), 937
Liquid biopsy-based clinical tools, 544
Liquid chromatography (LC), 173, 314
Liquid chromatography-mass spectrometry, 794, 795
Liquid chromatography tandem mass spectrometry (LC-MS), 402
Liver, 287, 300, 301, 303–305, 307
  enzymes, 210, 211
  glycogen, 1122
Liver-type fatty acid-binding protein (L-FABP), 900
Long non-coding RNAs (lncRNA), 599
Long QT syndrome, 815
Long term HFD-STZ induced Type 2 DM in wistar albino rats, 1129
Low-density lipoprotein (LDL), 397, 438, 952
Low-density lipoprotein cholesterol (LDL-C), 24, 137
Low-to-middle-income countries (LMICs), 518
LPL (Lipoprotein lipase) in the blood vessels hydrolysis monoglycerides and FFA, 459
Lysine-reactive chemicals, 997
Lysophosphatidylcholines, 795

M
Machine learning-based feature selection, 551, 553
Macroalbuminuria, 316, 801
2-Macroglobulin (A2MG), 335
Macrovascular and microvascular events associated with T2D, 549
Macrovascular (cardiovascular and cerebrovascular diseases), 241
Macula, 984
Magnesium (Mg), 288
Magnetic resonance imaging (MRI), 829, 841, 843
Major urinary proteins (MUPs), 802
Malignant arrhythmias, 817
Malondialdehyde (MDA), 134, 1095
Mammalian target of rapamycin (m-TOR) protein, 489
Mass spectrometry (MS), 173, 314, 405, 1008
  analysis, 804
Maternal, fetal and neonatal complications of gestational diabetes mellitus, 1024
Maternal glycemic status, 1027
Maternal hormones, 385
Matrix metalloproteinase 9 (MMP-9), 285
Matrix metalloproteinases (MMPs), 317
Matsuda index, 354
Maturity-Onset Diabetes of the Young (MODY), 400
McAuley index, 355
Mean corpuscular volume (MCV), 11
Mean plasma glucose levels, 1026
Mechanical and thermal withdrawal latency tests (MWT and TWL), 1096
Mendelian randomization, 16
Mesenchymal stromal cells (MSCs), 566, 567, 569
Messenger RNAs (mRNA), 599
Meta-analysis, 624
Metabolic alterations, 241
Metabolic complications, 951, 952
Metabolic derangements in type 2 DM, 1115
Metabolic diseases, 241, 755, 757

Metabolic disorders, 562
Metabolic-dysfunction-associated fatty liver disease (MALFD), 197
Metabolic endotoxemia, 941
Metabolic markers, 241
Metabolic memory, 286
Metabolic syndrome (MetS), 74, 162, 163, 199, 220, 321, 322, 325, 1080, 1082
Metabolism, 282–284, 301–303, 305, 307
Metabolites, 240, 982
  urinary profile, 804
Metabolomics, 1035, 1039–1047
Metformin, 564, 797
Methacholine-induced respiratory discomfort, 461
Methionine, 282
Methylation, 598–601, 607, 609
Methylation-specific PCR, 600
Methylglyoxal, 183
3-Methylhistidine, 904
Methylmalonic acid (MMA), 304
Methylmalonyl semialdehyde (MMS), 302
5′-Methylthioadenosine, 795
Microalbuminuria, 414, 895, 898, 904
Microaneurysms, 968
Microglia, 1096
Microhemorrhages, 689
Micronutrient, 288
Microorganisms, 927
MicroRNAs (miRNAs), 599, 650, 903, 982
  biogenesis, 652, 657
  inflammatory and endothelial dysfunctions, 651
  non-invasive biomarkers, 652
  post-transcriptional expression of target genes, 651
Microvascular complications, 549, 912
Microvascular (retinopathy and nephropathy), 241
Mild cognitive impairment (MCI), 323
miR-1281, 652, 660–662
Mitochondria, 302–305
Mitochondrial-DNA, 676
Mitochondrial dysfunction, 287
Mitogen-activated protein kinase (MAPK), 285, 1104
Moderate continuous training (MCT), 33
Molecular biology, 545
Molecular docking, 1129
  studies, 1130
Monocyte chemoattractant protein-1 (MCP-1), 872, 873, 1100, 1101
Monocyte chemotactic protein 1 (MCP-1) synthesis, 442
Monomethyl branched-chain fatty acids (mmBCFA), 118
Monounsaturated fatty acids (MUFA), 439, 452
Mucus layer, 927
Multiple regression analysis, 618, 623
Multiple sclerosis, 578–579
Myelin, 177
Myocardial blood flow, 824, 832, 835, 837, 840, 841, 843, 844
Myocardial extracellular volume, 828, 844
Myocardial fibrosis, 823–825, 828, 829, 832, 835, 840, 841, 843–845
  applications, 841
  cardiac fibrosis, 835, 836
  extracellular volume, 835, 836
  longitudinal studies, 840
  serum biomarkers, 841
Myocardial vascular rarefaction, 823
Myokines, 47, 72
Myostatin (GDF-8), 225

N
n-3 and n-6 PUFA, 453
n-3-desaturases, 453
n-6 PUFA (arachidonic acid)-induced proinflammatory prostaglandin E2 (PGE2) formation, 455
N-acetyl-β-D-glucosaminidase (NAG), 900
N-acetylneuraminate, 904
NADPH oxygenase-2 (NOX2), 1100
Nano-hmC-Seal technology, 548
National Health and Nutrition Examination Survey (NHANES), 12
National Institute for Health and Care Excellence (NICE) guideline, 1027
Nephrin, 565
Nephropathy, 904
Nerve conduction velocity (NCV), 1095
Nerve growth factor (NGF), 504
Neurodegeneration, 1058, 1059
Neurofilamentous axonal neuropathy, 999
Neurohormonal dysregulation, 380
Neuroinflammation
  diabetic animal models, 1063–1064
  neuroinflammatory biomarkers, 1057, 1065
  pro-inflammatory biomarkers, 1058, 1063
Neurologic disorders, 322, 323
Neuronal plasticity, 1057
Neuron-glia crosstalk, 1098
Neuropathy, 313, 325, 1106
Neurotrophin, 502, 504
Neutrophil gelatinase-associated lipocalin (NGAL), 898

Nicotinamide adenine dinucleotide phosphate (NADPH), 1094
Nicotinamide adenine dinucleotide phosphate oxidase (NOX), 1103, 1104
Nicotinamide (NA), 793
Nitric oxide (NO), 158, 869, 870, 1094
NLRP3 inflammasome, 267
N-methyl D-aspartate (NMDA) receptor, 285
N-methyl-D-aspartate receptors (NMDARs), 1096, 1102
Nociceptive, 1106
NOD-like receptor family pyrin domain containing 6 (NLRP6), 929
NOD-like receptor protein 3 (NLRP3), 266
Non-albuminuric, 904
Nonalcoholic fatty liver disease (NAFLD), 25, 198–201, 220, 303, 323, 931
Non-continuous glucose monitoring (NCGM), 1024, 1025
Non-continuous versus continuous glucose monitoring, 1024
Nonenzymatic glycosylation, 1003
Non-esterified fatty acids (NEFA), 222
Non-insulin dependent diabetes mellitus (NIDDM), *see* Type 2 diabetes
Non-invasive, 335
Non-ischemic heart disease, 823
Non-nucleoside reverse transcriptase inhibitors (NNRTIs), 565
Non-proliferative diabetic retinopathy (NPDR), 418, 781
Normal glucose tolerance (NGT), 160
NO synthase (NOS), 158
Nro-inflammatory proteins, 175
Nuclear Factor Kappa B (NF-κB), 927
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), 1097, 1099, 1100
Nuclear magnetic resonance (NMR), 404, 1008
Nucleus of tractus solitarius (NTS), 753
Nutrient components, 927
Nutrition, 286

O

Obesity, 23, 24, 44, 72–74, 129, 133, 155, 199, 287, 300–302, 304–306, 317, 320, 460, 461, 560, 761, 955
Obesity-related metabolic disorders, 196
Obstructive sleep apnea (OSA), 759
Occupational *n*-hexane/2-hexanone neuropathy, 1008, 1009
Office blood pressure (OBP), 707
Office blood pressure measurement (OBPM)
  devices, 735, 736
  European guidelines, 739
  hypertension, 738
  measurements per occasion, 736, 737
  microalbuminuria, 738
  technique, 737, 738
  types, 734, 735
  US guidelines, 739
Ohasama study, 707
Omega-3 mediated systemic effects in metabolic pathways, 459
Omega-3 PUFA effects on Insulin sensitivity, 456
Omega-6/Omega-3 ratio, 457, 458
  in type 2 diabetes mellitus, 458–460
Omega fatty acids and their metabolites, 462
Omic technologies, 1142
Open-label study, 713
Optical coherence tomography (OCT), 969
Optical coherence tomography angiography (OCTA)
  deep capillary plexus, 780
  features of diabetic retinopathy, 780, 781, 783, 784
  limitations, 785
  principles of, 778, 779
  visualization methods, 779
Optic disc, 984
Oral glucose tolerance test (OGTT), 117, 353–355, 377, 521, 632, 762, 763, 769, 1038
Orthogonal partial least squares discriminant analysis (OPLA-DA), 795–798, 804
Otsuka Long-Evans Tokushima Fatty (OLETF), 1092
oxBS-Seq protocol, 547
Oxidative markers, 1125
Oxidative stress, 129, 183, 184, 285, 436, 437, 827, 966, 1124, 1125
  and endothelial dysfunction, 249
  and inflammatory responses, 1061
4-Oxopentanoate, 904
5-Oxoproline, 904

P

Pancreatic cancer, 322
Pancreatic β-cell dysfunction, 1115, 1122
Panretinal photocoagulation (PRP), 970
Paraoxonase 1 (PON1)
  application, 443
  cardiovascular complications, 443
  functions, 439, 440

Paraoxonase 1 (PON1) (*cont.*)
glucose metabolism, 439
physiological effects, 440
physiological roles, 439, 440
polymorphisms, 440, 441
single nucleotide polymorphism, 441
structure, 438
type 2 diabetes mellitus (T2DM), 441, 442
Paraoxonase (*PON*) gene family, 437
Parathion, 438
Parkinson's disease (PD), 293, 334
Pars plana, 984
Pathogen associated molecular patterns (PAMPs), 932
Pathogenicity, 586
Pathophysiology, 1106
Paw withdrawal threshold (PWT), 1096
Pediatric, 734, 736, 739, 740, 742
Pentosidine, 174, 902
Pericytes, 967
Perilipin protein 5 (PLIN5c), 31
Peripheral and central nervous systems, 1096, 1097
Peripheral artery disease (PAD), 321
Peripheral artery tonometry, 856, 857
Peripheral blood, 561
Peripheral circulation, 827
Peripheral nervous system (PNS), 487
Peripheral neuropathy, 994
Peripheral neuropathy in DM-2, 998
Peroxisome proliferator activated receptor-gamma (PPAR-γ), 73, 1095
Peroxisome proliferator-activated receptors (PPAR), 222
Persistent hyperglycemia, 263
Personalization of disease management, 1143
Pharmacodynamics, 532
Pharmacological DPP-4 inhibitors, 205, 206
Phenylalanine, 795
Phosphatidylinositol-3-kinase (PI3K), 489, 490
Phosphoinositide 3-kinase (PI3k), 637
Phospholipase C (PLC), 634
5-Phosphoribosyl-1-pyrophosphate (PRPP), 131
Phosphotidinositol-specific phospholipase C (PLCγ), 489
PI3K/Akt signalling pathway, 76
Pigment epithelium-derived factor (PEDF), 197, 209, 210, 977
Pimelate, 904
Placenta growth factor (PIGF), 489, 971
Planar cell polarity (PCP) pathway, 634
Plasma glucose concentration, 438
Plasminogen activator 1, 177
Podocalyxin, 565
Podocytes, 568
and interstitial vessel cells, 269
Podocytes-podocalyxin, 896
Polar lipids, 26, 32
Polymorphism, 401, 444
Polyphenols, 929
Polyunsaturated fatty acids (PUFAs), 452, 453
composition and cell membrane, 454
in cellular function, 454–456
Polyuria, 794
Population-based Casale Monferrato Study, 250
Population-based cohort study, 1079
Postprandial versus preprandial glucose monitoring, 1025–1027
Povary syndrome (PCOS), 224
PPARs (peroxisome proliferator-activated receptors), 1126, 1128
Precision medicine, 984
Prediabetes, 241, 253, 803
AD, 71
biochemical markers, 763, 764
chemosensitivity, 769
definition, 70
Dejour test, 764
diagnostic criteria, 70
early diagnosis, 761, 762
hyperinsulinemia, 761
new medical device, 764, 765, 767
obesity, 761
questionnaires, 762, 763
scales, 762, 763
Predictive biomarker, 984
Preeclampsia, 568
Pregnancy, 1034, 1035, 1037, 1038, 1040, 1042–1047
Prehaptoglobin 2, 695
Primary progressive MS, 579
Primary urine, 626
Prognostic biomarker, 984
Pro-inflammatory cytokines, 137, 266, 1059, 1060, 1064
Proliferative diabetic retinopathy (PDR), 413, 783, 784
Proliferative retinopathy, 969
Prostate cancer, 76
Protease-activated receptor 2 (PAR2), 203
Protectins, 455
Protein cross-linking, 1005, 1006
Protein kinase C (PKC), 27, 954
pathway, 893
Protein metabolism, 242

Protein pyrrole adducts, 999, 1000, 1003
Protein pyrrole formation, 999
Proteinuria, 417
Proteomic(s), 325
Proteomic biomarkers
  acute and chronic disorders, 325
  applications, 324
  biomarker, 313
  diabetes mellitus, 325
  immuno-based assays, 313
  personalized medicine, 324
  proteins, 313
  translational control, 313
Proximity extension assay (PEA), 974, 975
Pulmonary disorders, 461
Purified fraction of SC (L.) Skeels (SC2), 1121
Purine de novo biosynthesis, 147
Purines, 131
Purinosome, 129, 131, 146
Pyrimidines, 138
Pyrraline, 174
Pyrraline (epsilon 2-(formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine), 1006
Pyrrolecarboxyaldehydes, 1004
Pyrrole generation, 999
Pyrrole protein adducts, 1007, 1008

Q
QT interval
  atrial fibrillation, 814
  diabetes mellitus, 814
  dispersion, 815
  glycemic variability, 812, 813
  sudden cardiac death (SCD), 814
Quantitative insulin sensitivity check index (QUICKI), 353
Quantitative PCR (qPCR), 600

R
RAAS blocker at bedtime, 714
RAAS blockers, 708, 711
Rac-GEF pathway, 472
Randomized controlled trials (RCTs), 39, 1077
Rate of depression (RDD), 1103
Rat model
  adiponectin, 1095, 1096
  applications, 1106
  diabetes mellitus (DM), 1091
  diabetic neuropathy (DN), 1091–1093
  glycemic management, 1092
  oxidative stress markers, 1094, 1095
  proximal nerves, 1092
Reactive center loop (RCL), 157
Reactive hyperemia, 854
Reactive oxygen species (ROS), 9, 158, 183, 285, 302, 519, 1093, 1094
Reasons for Geographic and Racial Differences in Stroke (REGARDS) study, 815
Receiver operating characteristics (ROC), 338, 661
Receptors for AGEs (RAGEs), 966
Red blood cells (RBCs), 519
Redox-regulating protein, 903
Reduced optic nerve head (ONH) vascularity, 783
Refeeding-induced fat and liver (RIFL), 381
Reference intervals (RIs), 523
Remitting MS, 578
Renal biomarkers and diabetic retinopathy, correlations, 413, 414
Renal biomarkers and macular edema, correlations, 415, 416
Renal Biomarkers *vs*. Cataract in Diabetes, 423
Renal Biomarkers *vs*. Diabetic Keratopathy, 421
Renal Biomarkers *vs*. Glaucoma in Diabetes, 423
Renal function, 252
Renal impairment, 251, 252
Renal inflammation, 264
Renal Insufficiency and Cardiovascular Events Italian Multicentre (RIACE) study, 712
Renal replacement therapy, 420, 422
Renal Replacement Therapy or Kidney Transplantation on Cataract, 424
Renal Replacement Therapy or Kidney Transplantation on Glaucoma, 423
Renin-angiotensin-aldosterone system (RAAS), 249, 917
Renovascular disease, 568
Resistance, 23, 25, 42
Resistin
  biomarker, 72
    in cancer, 75
    in cardiovascular disorders, 73
    in diabetes, 74, 75
    in obesity, 73, 74
  cysteine-rich protein, 69
  regulatory roles, 77
  vs. pre-diabetes, 76, 77
Resistin-like molecules (RELMs), 72
Resolvins, 455
Restless leg syndrome (RLS), 322, 325
Retinal capillaries, 967

Retinal neovascularization, 968, 971, 979, 983
Retinal pigment epithelial cells (RPE), 978
Retinal vascular cells, 967
Retinol-binding protein-4 (RBP4), 226
Retinopathy, 313, 314, 324, 523, 731
Rheumatoid arthritis (RA), 695
Risk prediction, 398–401, 404, 405
RNA-duplex, 657
rs9934336, 623, 624

S
S-adenosylhomocysteine (SAH), 283
S-adenosylmethionine (SAM), 282
Salivary biomarkers, 336
Satellite glial cells (SGCs), 1096
Saturated fatty acids (SFAs), 452
Saxagliptin, 564
Scottish Intercollegiate Guidelines Network (SIGN), 1028
Secondary progressive MS, 579
Secreted frizzled receptor 4 (sFRP4), 633
Secreted frizzled related protein 4 (sFRP4), 642, 644
Secreted frizzled related protein 5 (sFRP5), 642, 644
Secreted frizzled-related protein (sFRP) family, 633, 634, 643
Secretory immunoglobulin A (sIgA), 929
Sedentary lifestyle, 286
Selenium (Se), 288
Selenium and risk of diabetes
  clinical studies, 1077–1078
  diabetes complications, 1079–1080
  gestational diabetes, 1078–1079
  glucose homeostasis, 1080–1081
Selenium and Vitamin E Cancer Prevention Trial (SELECT), 1077
Selenoprotein (SeP), 199, 1076, 1077, 1080, 1081
Selenoprotein P (SeP), 207, 1076, 1077, 1080
Self-monitoring of blood glucose, 1028
Self-reported ethnicity, 676
Sensitisation, 1106
Serine protease, 164
Serpin A12 (vaspin)
  adipokines, 155
  adipose tissue, 155, 157
  anti-inflammatory effects, 158, 159
  applications, 163, 164
  biochemical characterization, 157
  biological processes, 155
  biomarkers, 156, 157, 164
  cellular level, 159
  diabetes, 156
  glucose intolerance, 159–161
  hyperglycemia, 164
  inflammatory skin diseases, 158
  insulin resistance, 156
  insulin role, 161, 162
  metabolic syndrome (MetS), 162, 163
  obesity, 155, 162
  type 2 diabetes mellitus (T2DM), 162
  type 2 diabetes (T2D), 156, 157
Serum amino acid levels, 242
Serum biomarkers, 841
Serum creatinine, 413, 414
Serum cystatin, 900
Serum insulin and C peptide levels, 1121
Serum leptin
  GH/IGF-1 increases, 953
  medical therapy, 957
  medical treatment, 959
  metabolic effects, 952
  metabolic process, 953
Serum lipid control, 970
Serum molecular biomarkers in diabetic neuropathy, 999
Serum NGAL, 899
Serum uric acid
  blood glucose level and HbA1c, 249
  level with diabetes and complications, 246–248
Sex, 618, 620, 623, 626
SGLT2 inhibitors, 624–626
Sheer stress, 851, 854
Short-chain fatty acids (SCFAs), 926, 929
Shortened MOLLI (ShMOLLI), 831
Short QT syndrome, 812, 817
Short tandem repeats (STRs), 673
Sickle cell disease, 11
Sickle cell trait, 16
Sight threatening DR (STDR), 413
Silent myocardial infarction (SMI), 815
Singapore Epidemiology of Eye Diseases Study, 676
Single nucleotide polymorphisms (SNPs), 139, 159, 400, 440, 622, 626, 672–675, 812
Single-nucleotide variations (SNVs), 904
Skeletal muscle, 287, 301–303, 307, 561
Skeletal muscle mass (SMM), 74
Skin, 461
  autofluorescence, 860
Smooth muscle cells, 158

Society of Canadian Obstetrics and Gynecology, 1028
Sodium dedocyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 799
Sodium glucose channel inhibitors (SGLT2i), 565
Sodium-glucose co-transporter-2 (SGLT-2) inhibitors, 420
Sodium-glucose transporter 2 (SGLT2) inhibitors, 616
Sodium-glucose transport protein 2 (SGLT2), 626, 823
Soluble RAGE (sRAGE), 175
Solute carrier 5A2 (SLC5A2), 621–626
Solute carrier 5 (SLC5), 620
Somatostatin, 951
Sphingolipids, 31, 32
Splicing of betatrophin transcripts, 388
Spontaneously diabetic torii (SDT), 1093
Statistical probability modeling approach, 676
Stawiski calculator, 360
Stem cells, 569
Sterol regulatory element binding protein 1c ($SREBP_{1c}$), 399
Streptozotocin (STZ), 793, 1092, 1116
Stress oxidative, 474, 477, 481
Stroke, 560
Structure toxicity relationship of aliphatic γ-diketones, 1001
Stumvoll index, 355
Sudden cardiac death (SCD), 817
Sulfhydryl groups, 282
Superoxide anion (O_2^-), 1094
Supervised exercise training (SET), 563
Supplementation for diabetic retinopathy prevention, 917, 918
Suppressor of cytokine signaling 3 (SOCS3), 44
Sustained hyperglycemia, 241
Sympathetic nervous system (SNS), 753
Systemic lupus erythematosus (SLE), 333
Systolic blood pressure (SBP), 134
Syzygium cumini (SC), 1113, 1129, 1130

T

T1 mapping technique, 829
T2D-assocaited vascular complications, 551
T2D-associated complications, 543, 544, 549
TAB-Seq protocol, 546
TargetScan bioinformatic prediction algorithm, 662
T cell/B cell activation molecule (TBAM), 580
T cell factor (TCF), 634
T cell receptor revision, 582, 583, 585, 589
TG-to-HDL-C ratio, 356
Th40 cells, 579–583
  generation, 581
  inflammatory cytokines, 583
  in multiple sclerosis, 587–588
  in T1DM, 586–587
Thermodynamics and kinetics of pyrrole adduction, 1008
Thiazolidinediones (TZDs), 72
Thioredoxin reductase (TXNRD), 1076
Thyroid hormones, 284
Tight junction proteins, 929
Tissue inhibitor of metalloproteinase 4 (TIMP-4), 285
Tissue T1 times, 830, 831
Toll-like receptor 4 (TLR4), 203, 927
Toll-like receptors (TLRs), 42, 55, 265, 1097
Total cholesterol (TC), 73, 137, 290
Transcription factor NF-kappaB (NF-κB), 177
Transient receptor potential vanilloid-1 receptor (TRPV1), 1100, 1101
Trans-Omics for Precision Medicine Program (TOPMed), 5
Traumatic brain injury (TBI), 567
Triacylglycerol-enriched remnant lipoproteins (TRL), 397, 398
Triacylglycerol (TG), 397, 399, 401, 951
TrialNet, 586
Tricarboxylic acid cycle (TCA), 90, 301
Tricine gel electrophoresis, 799
Triglycerides (TG), 73, 133, 290
Triplet proteins of 10nm neurofilaments, 1004
TRL particle size, 398–400, 402, 404, 405
Tsukushi (TSK), 210
Tubular damage and inflammation, 263
Tubulin, 177
Tubulointerstitial damage, 271
Tumor necrosis factor (TNF), 73
Tumor necrosis factor-α (TNF-α), 41, 639, 931, 1095, 1100, 1125
  pathways, 399
Two-dimensional polyacrylamide gel electrophoresis (2D PAGE), 314
Type 1 diabetes (T1D), 165, 598, 670, 672, 674, 675, 730, 1056
  experimental model, 1063
  uric acid, 254
Type 1 diabetes mellitus (T1DM), 518, 576, 631
  auto-antibodies, 578
  biomarkers, 577–578

Type 2 diabetes (T2D), 156, 157, 165, 300–302, 304–307, 334, 398–402, 404, 405, 560, 565, 598, 670, 671, 674–676, 730, 1038, 1056, 1057
and selenium, 1077–1081
Type 2 diabetes mellitus (T2DM), 23, 24, 29–31, 33, 34, 49, 55, 87, 129, 200, 220, 289, 294, 313, 314, 318, 324, 325, 436, 444, 470, 474, 475, 478, 518, 631, 938, 941, 1113
bacterial translocation, 925
cardiovascular diseases, 320, 321
gut barrier, 925
gut dysbiosis, 925
insulin resistance, 954, 955
intestinal barrier, 934
leptin, 954, 955
leptin resistance, 956
mechanism, 934
metabolic syndrome (MetS), 321, 322
microbiome research, 925
neurologic disorders, 322, 323
obesity, 317, 320, 925
pancreatic cancer, 322
peripheral organs, 925
Type IV collagen, 896
Tyrosine kinase, 1103

U
UK Prospective Diabetes Study (UKPDS), 524, 970
Ultra-widefield photography, 969
United Kingdom Prospective Diabetes Study (UKPDS), 417
Unsaturated fatty acids, 452
Uremic cardiomyopathy, 178
Uric acid (UA), 133, 141–143, 242, 243, 903
as biomarker in diabetes, 245
in development of abnormal glucose metabolism, 245
in development of diabetic complications, 250
in diabetes pathophysiology, 243–245
in diabetic complications, 249
in macrovascular complications, 250, 251
in microvascular complications, 251, 252
in pathophysiology of type 2 diabetes mellitus, 244
Urinary glucose excretion
diabetes mellitus, 616, 617
diabetic state, 618–620
glucose homeostasis, 617
glucosuria, 617, 618
kidney and transport glucose, 616
SGLT2 gene, 621
sodium-glucose cotransporters, 620, 621
Urinary interleukins, 267
Urinary metabolites, 793, 795–797, 804
Urinary profiling
animal model, 793, 794
blood glucose level, 803
diagnosis specimen, 793
dietary control, 797, 799
histone H4, 801–803
human disease, 793, 794
liquid chromatography-mass spectrometry, 794, 795
metabolite profiles, 803
non-invasive method, 804
physical activity, 797, 799
pre-diabetes, 792, 803, 804
protein biomarkers, 800, 801
protein list, 802
proteomics analysis, 799
separation and analysis techniques, 804
tricine SDS-PAGE, 801, 802
Urinary protein profile, 804
Urinary proteins, 793, 799, 801
Urine and serum IL-18 concentrations, 271
US Diabetes Prevention Program (DPP), 561
U-shaped metabolic effect, 1082

V
Val-derived metabolite, 118
Vascular cell adhesion molecule (VCAM-1), 517
Vascular disease, 290
Vascular endothelial cells, 158, 978
Vascular endothelial growth factor (VEGF), 175, 564, 967, 983, 1105
expression, 1060
Vascular endothelial growth factor A (VEGFA), 489, 971
arteriogenesis, 490
atherosclerosis, 490
cardiomyocytes, 490
diabetes complications, 497–499
diabetic polyneuropathy, 499–502
endothelial cells, 490
gene promoter, 662
growth factors, 496, 497
hypoxia, 489
intracellular signalling reactions, 491
lymphangiogenesis, 489

receptors, 490
type 1 diabetes mellitus, 496, 497
type 2 diabetes mellitus, 495, 496
tyrosine kinase activity, 489
Vascular endothelial growth factor receptor (VEGFR2), 501
Vascular-related diseases, 474
Vascular smooth muscle cells (VSMCs), 158
Vasomotor function, 852
Venous occlusion plethysmography, 852–854
Very-low-density lipoproteins (VLDL), 24, 404
Visceral adipose tissue (VAT), 74, 165, 198, 200
Visceral adiposity index (VAI), 356
Vitamin(s), 288
Vitamin B, 228, 229
Vitamin B12, 284
Vitamin B6, 284
Vitamin C, 228
Vitamin D, 228, 229, 910
deficiency, 910, 911, 918
and diabetes mellitus, 912
and diabetic retinopathy development and severity, 912, 913
diabetic retinopathy pathophysiology, 913, 917
sufficiency, 911
supplementation for DR prevention, 917
Vitamin E, 228
Vitamin K, 228, 229
Vitreous biomarkers
applications, 982, 983
chemokines, 974
clinical grading, 969
Detection, 971
DR biomarkers, 972
growth factors, 974
method of analysis, 973, 974
ocular liquid biopsy, 972
pathogenesis, 968
pathophysiology, 966–968
sample selection, 973, 974
VLDL1, 398–402, 405

W
w-3 and w-6 PUFA, 452
Waist circumference (WC), 133
Water surface tension, 799
Weight loss, 364
Western blotting, 177
Westernized lifestyle in developing countries and the epidemic of obesity, 543
White adipocyte, 305
Whole genome sequencing (WGS), 5, 9, 16
Wide-field OCTA, 785
Williams formula, 357
Wilm's tumor, 565
Wnt/beta-catenin pathway, 642
Wnt/non-canonical pathways, 642
Wnt signaling pathway, 642
World Health Organization (WHO), 763

X
Xanthine oxidase (XO), 146, 147
glycemic parameters, 134
MDA and superoxides, 134
metalloflavoenzyme, 133
pathological conditions, 133
positive association, 133, 134

Z
Zheng formula, 360
Zinc (Zn), 288
Zonulin, 695