R. C. Sobti
Awtar Krishan Ganju *Editors*

Biomedical Translational Research

From Disease Diagnosis to Treatment

Springer

Biomedical Translational Research

R. C. Sobti • Awtar Krishan Ganju
Editors

Biomedical Translational Research

From Disease Diagnosis to Treatment

Springer

Editors
R. C. Sobti
Department of Biotechnology
Punjab University
Chandigarh, India

Awtar Krishan Ganju
Department of Pathology
University of Miami Health System
Florida, USA

ISBN 978-981-16-8844-7 ISBN 978-981-16-8845-4 (eBook)
https://doi.org/10.1007/978-981-16-8845-4

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

The volume is dedicated to the respected parents of the Editors for their blessings from heaven

Preface

The basic biomedical research aims to provide a comprehensive and detailed understanding of the mechanisms that underlie the development and normal functions of humans and other living organisms. Moreover, organismal physiology has been a most significant challenge ahead in basic and clinical research. Attempts are on to understand the integrated function of organs and organisms. The recent researches have facilitated to understand the disease-causing pathological and pathophysiological mechanisms. There have been remarkable conceptual and technical advances in biological and biomedical sciences in the last few years and are continuing rapidly. The genome project and developments of OMICS technologies in combination with computational and imaging technologies have provided new language to the understanding of occurrence, mechanism, and prevention of disease. Now molecular mechanisms of many acquired and inheritable diseases have been elucidated. The mysteries of the brain are being unravelled for the study of cells, organs, and patients. Though there has been an explosion of information in all these areas, it is difficult to collate all that for practical uses. There is, thus, a wide gap in knowledge and its applications. To mitigate the challenges faced by humans, this gap must be bridged. There is a dire need to have an effective dialog between physicians and scientists. It will help in understanding clinical medicine in a much practical way. The interaction of astute clinicians with patients may stimulate clinical investigations that may suggest novel mechanisms of disease. There is, in fact, a bidirectional flow of information from patients to the laboratory and back. It helps to accelerate understanding of human diseases and develop new strategies to prevent, diagnose, and treat them. Its route may pass through various experimentation and validation stages in lower and higher animal species and now on chops, cell-free systems, and bionomics. There can be no doubt that the frequency and intensity of interactions have tremendously increased now. The primary and clinical workforces linked by biomedical scientists are now also termed "translational" researchers. They are trained to be knowledgeable in the primary and clinical biomedical sciences and proficient in patient care.

The volume "Translational Biomedical Sciences" is a platform for clinical researchers, basic scientists, biomedical engineers, and computational biologists from the USA, Japan, Australia, Canada, Ta Singapore, and India to express their experiences and futuristic thoughts in the form of chapters.

It has been compiled in three volumes, i.e., it summarizes emerging technologies for healthcare. Volume II *From Disease Diagnosis to Treatment* discusses various aspects of biomedical research towards understanding the diseases' pathophysiology and improvement in diagnostic procedures and therapeutic tools. Volume III *Drug Design and Discovery* focuses on biomedical research's fundamental role in developing new medicinal products.

The second volume of biomedical translational research discusses various aspects of biomedical research towards understanding of pathophysiology of the diseases and improvement in diagnostic procedures and therapeutic tools. It presents integration of molecular-based technologies, systematic tissue procurement, and medical informatics that can be translated into useful diagnostic and treatment strategies. It discusses improvements in the translational research for discovery of new diagnostic tests identification of novel biomarkers and druggable targets, and prediction of optimal treatments based upon the underlying molecular basis of the disease.

Chandigarh, India — R. C. Sobti
Florida, USA — Awtar Krishan Ganju

Acknowledgements

R.C. Sobti is thankful to his wife Dr. Vipin Sobti and daughters Er. Aditi Sobti and Dr. Aastha Sobti (their spouses Er. Vineet and Er. Ankit) for their eveready support in preparing this volume. The encouraging words of his granddaughter Irene cannot be put in black and white.

R.C. Sobti acknowledges the Indian National Science Academy (INSA) for providing support under the Senior Scientist Programme of the Academy.

Contents

About the Editors

R. C. Sobti former Vice-Chancellor of Panjab University, Chandigarh and Babasaheb Bhimrao Ambedkar University (Central University), Lucknow, is a scientist, an able administrator, and dynamic institution builder.

Starting his career as a Cytogeneticist, he moved on to molecular biology including genomics to understand the susceptibility and disease process of cancer, COPD, AIDS metabolic syndrome, and kidney diseases. He has also used stem cells and nanoparticles to follow the process of tissue organ development through designed decellularization protocol. Earlier, he had worked on the cytogenetics and molecular genetics of various animal groups including humans as well as molecular toxicology of agricultural pollutants using *in vitro* and *in vivo* protocols.

He has published more than 300 papers in the journals of national repute such as Mutation Research, Carcinogenesis. Archives of Toxicology, Cancer Genetics and Cytogenetics, Molecular Cell Biochemistry, PLOS I, to name a few. He has also published more than 40 books by international publishers.

He is a Fellow of the Third World Academy of Sciences, National Academy of Sciences India, Indian National Science Academy, National Academy of Medical Sciences, National Academy of Agricultural Sciences, Canadian Academy of Cardiovascular Diseases, and few others. He was the General President of Indian Science Congress for the 102nd session held at the University of Jammu in 2013. Dr. Sobti is the recipient of many prestigious awards like the INSA Young Scientist Award, UGC Career Award, Punjab Rattan Award, JC Bose Oration and Sriram Oration Awards, and of Lifetime Achievement Awards of the Punjab Academy of Sciences, the Zoological Society of India, and the Environment Academy of India, besides many other medals and awards of national and International levels. Dr. Sobti, an active researcher, is also steadfastly committed to the popularization of science in the community through popular lectures and community engagement programs.

Awtar Krishan Ganju had been Scientific Director of Comprehensive Cancer, University of Miami Medical School. Now he is Emeritus Professor, Department of Pathology in the University of Miami, Medical School, Miami. He is a renowned cancer biologist and has been one of the pioneers in the flow cytometry cancer diagnosis, and drug development. He has published a large number of papers in the international journals of repute. He has widely traveled and has trained faculty and students in flow cytometry technology world over.

AGE-RAGE Stress in the Pathophysiology of Atherosclerosis and Its Treatment

1

Kailash Prasad

Abstract

AGE-RAGE stress induces atherosclerosis through the production of numerous atherogenic factors including reactive oxygen species (ROS), minimally modified low-density lipoprotein (MM-LDL), oxidized LDL (OX-LDL), monocyte chemoattractant protein-1, adhesion molecules, cytokines, monocyte colony-stimulating factor, growth factors, endothelin-1, and reduction in nitric oxide. All of the above factors except nitric oxide and endothelin-1 are involved in the oxidative hypothesis of atherosclerosis. Treatment of AGE-RAGE stress-induced atherosclerosis should be targeted at the reduction in AGE intake, prevention of AGE formation, degradation of AGE in vivo, suppression of RAGE expression, blockade of AGE binding with RAGE, elevation of sRAGE by increasing sRAGE expression and exogenous administration, and use of antioxidants. These treatment modalities would prevent, regress, and slow the progression of AGE-RAGE stress-induced atherosclerosis and hence would assist in the treatment of coronary artery disease and stroke.

Keywords

Atherosclerosis · Reactive oxygen species · Advanced glycation end product (AGE) · Receptor for AGE (RAGE) · Soluble receptor for AGE (sRAGE) · NF-kB · Cytokines · Adhesion molecules · Chemoattractant protein-1 · Growth factors · Endothelin · Nitric oxide · Prevention of AGE formation · Downregulation of RAGE, and sRAGE expression

K. Prasad (✉)
Department of Physiology (APP), College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada
e-mail: k.prasad@usask.ca

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_1

1.1 Introduction

Atherosclerosis is a disease of large- and medium-sized arteries and is characterized by focal thickening of the intima of the arterial wall and lipid deposition resulting in the hardening and narrowing of arteries. Atherosclerosis leads to cardiovascular diseases (CVDs) including ischemic heart disease (IHD), stroke, and peripheral vascular disease (PVD). The main forms of CVD are coronary artery disease (CAD) and stroke. Fifty percent of all deaths from CVD are due to CAD and 25% to stroke (Petersen et al. 2006). CAD also called CHD is the leading cause of death globally. The risk factors for CHD and stroke are similar and include dyslipidemia (World Health Organization 2002; Castelli 1988), diabetes (Kannel and McGee 1979), hypertension (Kennel 1975), cigarette smoking (Prasad 2000; British Heart Foundation 2017), obesity (Poirier et al. 2006), hyperhomocysteinemia (Glueck et al. 1995), and C-reactive protein (Prasad 2004).

Advanced glycation end products (AGEs) and its cell receptors for AGE (RAGE) and soluble receptors for AGE (sRAGE) and endogenous secretory receptor for AGE (esRAGE) have been implicated in various diseases including, non-ST-elevation myocardial infarction (McNair et al. 2009), restenosis following percutaneous coronary intervention (McNair et al. 2010), hyperthyroidism (Caspar-Bell et al. 2016), hypertension (Prasad and Mishra 2017), pulmonary hypertension (Prasad 2015), and aortic aneurysm (Prasad et al. 2016b; Prasad 2019). This book chapter addresses the AGE-RAGE axis, AGE-RAGE stress, and the role of AGE-RAGE stress in the pathogenesis of atherosclerosis and the treatment strategy for prevention, regression, and slowing down of the progression of atherosclerosis and associated cardiovascular diseases.

1.2 AGE-RAGE Axis

AGE-RAGE axis consists of AGE, RAGE, and sRAGE which comprises of esRAGE and cleaved RAGE (cRAGE). AGEs are a heterogeneous group of irreversible adducts generated by the nonenzymatic glycation and glycoxidation of proteins, lipids, and nucleic acid with reduced sugars (Thorpe and Baynes 2003; Bucala and Cerami 1992). There are three main receptors of AGEs: N-truncated RAGE (full-length RAGE) and C-terminal RAGE that has two isoforms, cleaved RAGE (cRAGE) and endogenous secretory RAGE (esRAGE). cRAGE is proteolytically cleaved from full-length RAGE (Tam et al. 2011) and esRAGE is generated from alternative messenger RNA splicing of full-length RAGE (Yonekura et al. 2003). sRAGE measurement includes both cRAGE and esRAGE and circulates in the blood. esRAGE is about 20–30% of sRAGE (Koyama et al. 2005; Prasad et al. 2016a). The interaction of AGE with RAGE generates the reactive oxygen species (ROS) via activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Wautier et al. 2001) which in turn activates the nuclear factor-kappa B (NF-kB) (Gloire et al. 2006) that stimulates numerous proinflammatory cytokine genes including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1, IL-2,

IL-6, IL-8, and IL-9 (Reznikov et al. 2004; Stassen et al. 2001). Proinflammatory cytokines are known to upregulate the expression of NADPH oxidase (Mohammed et al. 2013) which further increases the production of ROS (Yang et al. 2007). The interaction of AGE with RAGE also increases the generation of chemoattractant protein-1, growth factors, and adhesion molecules and will be discussed in detail in the section of cell receptor-mediated effects of AGE. sRAGE and esRAGE act as a decoy for RAGE by binding with RAGE ligand (Geroldi et al. 2006). sRAGE and esRAGE binding with AGE does not activate intracellular signaling.

1.3 AGE-RAGE Stress

Low levels of serum sRAGE (Falcone et al. 2005; McNair et al. 2009; Caspar-Bell et al. 2016) and esRAGE (Koyama et al. 2005; Katakami et al. 2005) have been considered as biomarker of diseases. But it is not a universal biomarker because the serum levels of sRAGE are elevated in diabetes (Fujisawa et al. 2013; Challier et al. 2005) and end-stage renal disease (Prasad et al. 2016a; Kalousová; et al. 2006). Higher levels of esRAGE have also been reported to be associated with incident coronary artery disease (Colhoun et al. 2011). Therefore, low serum levels of sRAGE and esRAGE cannot be a universal biomarker. AGE-RAGE axis comprises of three components: AGE, RAGE, and sRAGE. Therefore, all three components should be considered in the assessment of a universal biomarker. Prasad then proposed that AGE/sRAGE should be considered as a universal biomarker (Prasad et al. 2016a; Prasad 2019). Recently Prasad and Mishra (2018) have coined the terminology "AGE-RAGE stress" which takes into consideration stress (AGE, RAGE) and antistressors [sRAGE, degraders of AGE (AGE receptors 1 & 2, glyoxalase 1 and 2)]. AGE-RAGE stress has been defined as a shift in the balance between stressors and antistressors in favor of stressors. Prasad and Mishra (2018) have derived an equation using stressors and antistressors for AGE-RAGE stress. The simplified and feasible formula for clinical purpose is AGE/sRAGE. An increase in the ratio of AGE/sRAGE indicates an increase in the AGE-RAGE stress. AGE/sRAGE thus serves as the universal biomarker/risk marker.

1.4 How AGE-RAGE Stress Modulates the Risk Factors for Atherosclerosis

AGE-induced atherosclerosis is mediated through nonreceptor and receptor mechanisms. The details are described by Prasad and Bhanumathy (2020).

1.4.1 Nonreceptor-Mediated Effects of AGE

AGE makes LDL more atherogenic through modification of apoB100 (Bucala et al. 1994). It alters LDL clearance and increases the susceptibility of LDL oxidation (Brownlee et al. 1985; Bucala et al. 1993). Glycated LDL enhances smooth muscle cell proliferation and differentiation (Makita et al. 1999), decreases its recognition by LDL receptors (Haberland et al. 1992), and interferes with cholesterol transport (Horiuchi et al. 2003). Glycation increases synthesis of collagen (Striker and Striker 1996). Cross-linking of AGE on collagen and elastin increases extracellular matrix and hence increases arterial stiffness (Tanaka et al. 1988). Matrix-bond AGE enhances expression of endothelin-1 (Quehenberger et al. 2000) which has been involved in the development of atherosclerosis (Sutton et al. 2019). AGE decreases the generation of nitric oxide (NO) (Goldin et al. 2006) and quenches NO (Bucala et al. 1991). Oxidized LDL reduces generation of NO (Ren et al. 2017; Cominacini et al. 2001). Matrix-bond AGE reduces generation of NO (Xu et al. 2003), quenches and inactivates NO (Goldin et al. 2006), and inhibits antiproliferative effect of NO (Hogan et al. 1992). These data suggest that AGE promotes atherosclerosis through LDL oxidation, increasing extracellular matrix, interfering cholesterol transport, altering LDL clearance, increasing smooth muscle cell proliferation and differentiation, and increasing expression of endothelin.

1.4.2 Receptor-Mediated Effects of AGE

Interaction of AGE with RAGE generates reactive oxygen species (ROS) (Wautier et al. 2001) which activates NF-kB. NF-kB activates numerous inflammatory genes including TNF-Î±, TNF-Î², IL-1, IL-6, and IL-8 (Reznikov et al. 2004; Siebenlist et al. 1994). AGE interacts with RAGE to increase expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (Basta et al. 2002). ROS upregulates the expression of ICAM-1, VCAM-1, and endothelial leucocyte adhesion molecules (ELAMs) (Willam et al. 1999; Chiu et al. 1997; Fraticelli et al. 1996). AGE-RAGE interaction increases expression of monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor in human-cultured mesangial cells (Yamagishi et al. 2002). Expression and secretion of granulocyte macrophage-colony-stimulating factor (GM-CSF) by macrophages are increased with AGE (Sasaki et al. 1999). AGE interaction with RAGE induces expression of insulin-like growth factor (IGF), IGF-1, and platelet-derived growth factor (PDGF) (Kirstein et al. 1990, 1992). Expression of transforming growth factor-Î² (TGF-Î²) is enhanced by AGE (Wolf et al. 1994). Interaction of AGE with sRAGE has protective effects against adverse effects of AGE-RAGE interaction.

Nonreceptor and receptor-mediated effects of AGE are atherogenic while interaction of AGE with sRAGE is non-atherogenic.

1.5 Mechanism of AGE-RAGE Stress-Induced Atherosclerosis

AGE and its interaction with RAGE generate numerous atherogenic factors and mediators. Oxidative hypothesis for atherosclerosis has been accepted universally and has been described in detail by Prasad (2000). Oxidative hypothesis fits very well for AGE-RAGE-induced atherosclerosis. Initial step in this hypothesis of atherosclerosis is mild oxidation of LDL resulting in the formation of minimally modified LDL (MM-LDL) which is further oxidized to form oxidized LDL (OX-LDL). Monocyte adherence to endothelial cells is mediated by adhesion molecules (ELAM, E-selectin, VCAM-1, and ICAM-1), and after adherence the monocytes transmigrate into the subendothelial cells (Aronson and Rayfield 2002). OX-LDL also upregulates the ICAM-1 and VCAM-1 (Li et al. 2002). OX-LDL enhances monocyte migration into subendothelial space through increasing expression of MCP-1. Monocytes/macrophages entering subendothelial space have LDL receptor but the rate of uptake of native LDL is not sufficient to produce foam cells (Goldstein et al. 1979). OX-LDL is a ligand for scavenger receptor which is expressed in tissue macrophage differentiated from monocytes (Henriksen et al. 1983). Differentiation of monocyte/macrophage is increased by monocyte-CSF. Receptors for OX-LDL uptake develop in differentiated macrophages. OX-LDL is taken up by differentiated macrophages to form foam cells which are early stages of atherosclerosis. Numerous growth-regulating molecules generated by macrophages enhance smooth muscle cell proliferation and migration and fibrous tissue formation, migration of monocytes, and formation of foam cells resulting in the development and progression of atherosclerosis. Fatty streaks develop in full-fledged atherosclerosis.

1.6 Evidence for AGE-RAGE Stress-Induced Atherosclerosis

As described above, AGE and its interaction with RAGE produce numerous atherogenic factors. A question arises if AGE-RAGE axis is involved in the development of atherosclerosis. Literatures show that there are evidences suggesting the role of AGE-RAGE axis in the development of atherosclerosis. It has been reported that AGE and RAGE levels are elevated in the carotid arterial wall of Zucker diabetic rats as compared to euglycemic control rats and that the AGE and RAGE levels are further elevated in the balloon-injured carotid artery of these rats (Zhou et al. 2003). Administration of sRAGE before and for up to 21Â days post-balloon injury significantly reduced neointimal hyperplasia, and this was associated with decreases in vascular smooth muscle cell growth in vitro and vascular smooth muscle cell proliferation in vivo in these rats. Arterial de-endothelialization in wild-type mice has been shown to increase the expression of RAGE in injured vessel, especially in smooth muscle cells, and increased deposition of AGE in expanding intima (Sakaguchi et al. 2003). These investigators also demonstrated that the administration sRAGE decreased neointimal hyperplasia, smooth muscle cell proliferation and migration, and expression of extracellular matrix protein. Atherosclerosis was

accelerated in apoE-deficient mice by streptozotocin-induced diabetes, and this effect was associated with increased expression of VCAM-1 in aorta when compared to nondiabetic mice (Wendt et al. 2000). These investigators also reported that administration of sRAGE significantly decreased the atherosclerotic lesion in a glycemic- and lipid-independent manner. Kislinger et al. (2001) have reported that expression of RAGE and VCAM-1 was elevated in aorta of apoE-deficient diabetic rats and that expression of RAGE and VCAM-1 was downregulated with administration of sRAGE. Park et al. (1998) have reported that sRAGE completely suppressed accelerated and advanced atherosclerosis in apoE-deficient mice. McNair et al. (2009) have reported that sRAGE levels are reduced in patients with non-ST-elevation myocardial infarction. McNair et al. (2010) have also shown that reduced serum levels of sRAGE are a predictor of restenosis following percutaneous coronary intervention. The role of AGE and RAGE in the development and progression of carotid artery stenosis has been discussed in detail by Prasad et al. (2015). AGE-RAGE axis may also play a role in the development of coronary artery disease (Fishman et al. 2018). It is to note that ROS also depresses myocardial contractility (Prasad et al. 1993). This effect of ROS would add to the cardiac effects of atherosclerosis. ROS is involved in the development of atherosclerosis (Prasad 1999; Prasad and Kalra 1993; Yang et al. 2017).

1.7 Treatment of AGE-RAGE-Induced Atherosclerosis

Since AGE-RAGE axis is involved in the development of atherosclerosis, the treatment of atherosclerosis should be targeted at the reduction in AGE levels, suppression of RAGE expression, blockade of binding of AGE with RAGE, degradation of AGE in vivo, elevation of sRAGE, and use of antioxidants. These treatment modalities have been described in detail by Prasad and Bhanumathy (2020) and Prasad and Mishra (2017). Here I am describing the treatment of AGE-RAGE-induced atherosclerosis in brief. AGE levels in the body can be reduced by decreasing the dietary intake of food containing high levels of AGE such as red meat, cheese, cream, animal fat, and sweetened food (Weisenberger 2014). Individuals should be advised to use grains, legume, breads, vegetables, fruits, and milk which contain low amount of AGE (Uribarri et al. 2010). AGE degraders in vivo should be used to reduce the serum levels of AGE. However, they are not available for use in humans. Intake of AGE can be reduced by cooking food at low temperature in moist heat for a short period. Cooking at high dry heat (frying, broiling, grilling, and roasting) increases formation of AGE (Uribarri et al. 2010). Cigarette smoking should be avoided because it increases serum levels of AGE (Prasad et al. 2015). Sugar consumption should be reduced because sugar is involved in the formation of AGE.

There are agents (vinegar, lemon juice, benfotiamine, pyridoxine, vitamin C, vitamin D, vitamin E, Î±-lipoic acid, resveratrol, and curcumin) that can be used to prevent the formation of AGE (Prasad and Bhanumathy 2020). Statins, candesartan,

nifedipine, and rosiglitazone decrease the expression of RAGE (Prasad and Bhanumathy 2020).

sRAGE levels can be elevated by the upregulation of sRAGE expression and exogenous administration of sRAGE (Prasad and Bhanumathy 2020).

1.8 Conclusions

AGE-RAGE stress can induce atherosclerosis through the generation of numerous atherogenic factors. Treatment of AGE-RAGE stress-induced atherosclerosis includes the reduction of AGE intake, prevention of AGE formation, degradation of AGE in vivo, suppression of RAGE expression, blockade of AGE-RAGE binding, elevation of sRAGE, and use of antioxidants.

References

Aronson D, Rayfield EJ (2002) How hyperglycemia promotes atherosclerosis: molecular mechanisms. Cardiovasc Diabetol 1:1. https://doi.org/10.1186/1475-2840-1-1

Basta G, Lazzerini G, Massaro M et al (2002) Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 105:816–822

British Heart Foundation (Factfile 8/2001) (2017) Stopping smoking—evidence-based guidance. http://www.bhsoc.org/files/8213/4399/2345/bhf_factfile_aug_2001.pdf. Accessed 23 Jul 2017

Brownlee M, Vlassara H, Cerami A (1985) Nonenzymatic glycosylation products on collagen covalently trap low-density lipoprotein. Diabetes 34:938–941

Bucala R, Cerami A (1992) Advanced glycation: chemistry, biology, and implications of diabetes and aging. Adv Pharmacol 23:1–34

Bucala R, Tracey KJ, Cerami A (1991) Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J Clin Invest 87:432–438

Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H (1993) Lipid advanced glycosylation: pathway for lipid oxidation in vivo. Proc Natl Acad Sci U S A 90:6434–6438

Bucala R, Makita Z, Vega G et al (1994) Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. Proc Natl Acad Sci U S A 91:9441–9445

Caspar-Bell G, Dhar I, Prasad K (2016) Advanced glycation end products (AGEs) and its receptors in the pathogenesis of hyperthyroidism. Mol Cell Biochem 414:171–178

Castelli WP (1988) Cholesterol and lipids in the risk of coronary artery disease: the Framingham Heart Study. Can J Cardiol 4(Suppl A):5A–10A

Challier M, Jacqueminet S, Benabdesselam O, Grimaldi A, Beaudeux JL (2005) Increased serum concentrations of soluble receptor for advanced glycation end products in patients with type 1 diabetes. Clin Chem 51:1749–1750

Chiu JJ, Wung BS, Shyy JY-J, Hsieh HJ, Wang DL (1997) Reactive oxygen species are involved in shear stress-induced intercellular adhesion molecule-1 expression in endothelial cells. Arterioscler Thromb Vasc Biol 17:3570–3577

Colhoun HM, Betteridge DJ, Durrington P et al (2011) Total soluble and endogenous secretory receptor for advanced glycation end products as predictive biomarkers of coronary heart disease risk in patients with type 2 diabetes: an analysis from the CARDS trial. Diabetes 60:2379–2385

Cominacini L et al (2001) The binding of oxidized low-density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. J Biol Chem 276:13750–13755

Falcone C, Emanuele E, D'Angelo A et al (2005) Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. Arterioscler Thromb Vasc Biol 25:1032–1037

Fishman SL, Sonmez H, Basman C et al (2018) The role of advanced glycation end-products in the development of coronary artery disease in patients with and without diabetes mellitus: a review. Mol Med 24:59

Fraticelli A, Serrano CV Jr, Bochner BS, Capogrossi MC, Zweier JL (1996) Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. Biochim Biophys Acta 1310:251–259

Fujisawa K, Katakami N, Kaneto H et al (2013) Circulating soluble RAGE as a predictive biomarker of cardiovascular event risk in patients with type 2 diabetes. Atherosclerosis 227: 425–428

Geroldi D, Falcone C, Emanuele E (2006) Soluble receptor for advanced glycation end products: from disease marker to potential therapeutic target. Curr Med Chem 13:1971–1978

Gloire G, Legrand-Poels S, Piette J (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 72:1493–1505

Glueck CJ, Shaw P, Lang JE, Tracy T, Sieve-Smith L, Wang Y (1995) Evidence that homocysteine is an independent risk factor for atherosclerosis in hyperlipidemic patients. Am J Cardiol 75: 132–136

Goldin A, Beckman JA, Schmidt AM, Creager MA (2006) Advanced glycation end products sparking the development of diabetic vascular injury. Circulation 114:597–605

Goldstein JL, Ho YK, Basu SK, Brown MS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci U S A 76:333–337

Haberland ME, Fless GM, Scanu AM, Fogelman AM (1992) Malondialdehyde modification of lipoprotein (a) produces avid uptake by human monocyte-macrophages. J Biol Chem 267:4143–4151

Henriksen T, Mahoney EM, Steinberg D (1983) Enhanced macrophage degradation of biological modified low density lipoprotein. Atherosclerosis 3:149–159

Hogan M, Cerami A, Bucala R (1992) Advanced glycosylation end products block the antiproliferative effect of nitric oxide. Role in the vascular and renal complications of diabetes mellitus. J Clin Invest 90:1110–1115

Horiuchi S, Sakamoto Y, Sakai M (2003) Scavenger receptors for oxidized and glycated proteins. Amino Acids 25:283–292

Kalousová M, Hodková M, Kazderová M, Fialová J, Tesar V, Dusilová-Sulková S, Zima T (2006) Soluble receptor for advanced glycation end products in patients with decreased renal function. Am J Kidney Dis 47:406–411

Kannel WB, McGee DL (1979) Diabetes and cardiovascular disease. The Framingham study. JAMA 241:2035–2038

Katakami N, Matsuhisa M, Kaneto H et al (2005) Decreased endogenous secretory advanced glycation end product receptor in type 1 diabetic patients: its possible association with diabetic vascular complications. Diabetes Care 28:2716–2721

Kennel WB (1975) Role of blood pressure in cardiovascular disease: the Framingham Study. Angiology 26:1–14

Kirstein M, Brett J, Radoff S, Ogawa S, Stern D, Vlassara H (1990) Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. Proc Natl Acad Sci U S A 87:9010–9014

Kirstein M, Aston C, Hintz R, Vlassara H (1992) Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins. J Clin Invest 90:439–446

Kislinger T, Tanji N, Wendt T et al (2001) Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. Arterioscler Thromb Vasc Biol 21:905–910

Koyama H, Shoji T, Yokoyama H et al (2005) Plasma levels of endogenous secretory RAGE is associated with components of the metabolic syndrome and atherosclerosis. Arterioscler Thromb Vasc Biol 25:2587–2593

Li D, Chen H, Romeo F, Sawamura T, Saldeen T, Mehta JL (2002) Statins modulate oxidized low-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. J Pharmacol Exp Ther 302:601–605

Makita T, Tanaka A, Numano F (1999) Effect of glycated low-density lipoprotein on smooth muscle cell proliferation. Int Angiol 18:331–334

McNair ED, Wells CR, Qureshi AM, Basran RS, Pearce C, Orvold J, Devilliers J, Prasad K (2009) Low levels of soluble receptor for advanced glycation end products in non-ST elevation myocardial infarction patients. Int J Angiol 18:187–192

McNair ED, Wells CR, Qureshi AM, Basran RS, Pearce C, Orvold J, Devilliers J, Prasad K (2010) Soluble receptor for advanced glycation end products (sRAGE) as a predictor of restenosis following percutaneous coronary intervention. Clin Cardiol 33:678–685

Mohammed AM, Syeda K, Hadden T, Kowluru A (2013) Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic beta-cells: attenuation of oxidative and nitrosative stress by 2-bromopalmitate. Biochem Pharmacol 85:109–114

Park L, Raman KG, Lee KJ et al (1998) Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation end products. Nat Med 4:1025–1031

Petersen S, Peto V, Scarborough P, Rayner M (2006) 2005 coronary heart disease statistics—2005 edition. British Heart Foundation Health Promotion Research Group, Department of Public Health, University of Oxford

Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH (2006) American Heart Association, Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Circulation 113:898–918

Prasad K (1999) Reduction of serum cholesterol and hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside isolated from flaxseed. Circulation 99:1355–1362

Prasad K (2000) Pathophysiology of atherosclerosis. In: Chang JB, Olsen ER, Prasad K, Sumpio BE (eds) Textbook of angiology. Springer, New York, pp 85–106

Prasad K (2004) C-reactive protein increases oxygen radical generation by neutrophils. J Cardiovasc Pharmacol Ther 9(3):203–209

Prasad K (2015) Pathophysiology and medical treatment of carotid artery stenosis. Int J Angiol 24: 158–172

Prasad K (2019) AGE-RAGE stress play a role in aortic aneurysm: a comprehensive review and novel potential therapeutic target. Rev Cardiovasc Med 20:201–208

Prasad K, Bhanumathy KK (2020) AGE-RAGE axis in the pathophysiology of chronic lower limb ischemia and a novel strategy for its treatment. Int J Angiol 29(3):156–167

Prasad K, Kalra J (1993) Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E. Am Heart J 125:958–973

Prasad K, Mishra M (2017) Do advanced glycation end products and its receptor play a role in pathophysiology of hypertension? Int J Angiol 26:1–11

Prasad K, Mishra M (2018) AGE–RAGE stress, stressors, and antistressors in health and disease. Int J Angiol 27:1–12

Prasad K, Kalra J, Bharadwaj L (1993) Cardiac depressant effects of oxygen free radicals. Angiology 44:257–270
Prasad K, Dhar I, Caspar-Bell G (2015) Role of advanced glycation end products and its receptors in the pathogenesis of cigarette smoke-induced cardiovascular disease. Int J Angiol 24:75–80
Prasad K, Dhar I, Zhou Q, Elmoselhi H, Shoker M, Shoker A (2016a) AGEs/sRAGE, a novel risk factor in the pathogenesis of end-stage renal disease. Mol Cell Biochem 423(01/02):105–114
Prasad K, Sarkar A, Zafar MA, Shoker A, Moselhi HE, Tranquilli M, Ziganshin BA, Elefteriades JA (2016b) Advanced glycation end products and its receptors in the pathogenesis of thoracic aortic aneurysm. Aorta 4:1–10
Quehenberger P, Bierhaus A, Fasching P et al (2000) Endothelin 1 transcription is controlled by nuclear factor-kappa B in AGE stimulated cultured endothelial cells. Diabetes 49:1561–1570
Ren X, Ren L, Wei Q, Shao H, Chen L, Liu N (2017) Advanced glycation end-products decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. Cardiovasc Diabetol 16:52
Reznikov LL, Waksman J, Azam T et al (2004) Effect of advanced glycation end products on endotoxin-induced TNF-alpha, IL-1 beta and IL-8 in human peripheral blood mononuclear cells. Clin Nephrol 61:324–336
Sakaguchi T, Yan SF, Yan SD et al (2003) Central role of RAGE-dependent neointimal expansion in arterial restenosis. J Clin Invest 111:959–972
Sasaki T, Horiuchi S, Yamazaki M, Yui S (1999) Induction of GM-CSF production of macrophages by advanced glycation end products of the Maillard reaction. Biosci Biotechnol Biochem 63: 2011–2013
Siebenlist U, Franzoso G, Brown K (1994) Structure, regulation and function of NF-κ B. Annu Rev Cell Biol 10:405–455
Stassen M, Müller C, Arnold M et al (2001) IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-κ B is decisively involved in the expression of IL-9. J Immunol 166:4391–4398
Striker LJ, Striker GE (1996) Administration of AGEs in vivo induces extracellular matrix gene expression. Nephrol Dial Transplant 11:62–65
Sutton G, Pugh D, Dhaun N (2019) Developments in the role of endothelin-1 in atherosclerosis: a potential therapeutic target? Am J Hypertens 32:813–815
Tam XHL, Shiu SWM, Leng L, Bucala R, Betteridge DJ, Tan KCB (2011) Enhanced expression of receptor for advanced glycation end-products is associated with low circulating soluble isoforms of the receptors in type 2 diabetes. Clin Sci 120:81–89
Tanaka S, Avigad G, Brodsky B, Eikenberry EF (1988) Glycation induces expansion of the molecular packing of collagen. J Mol Biol 203:495–505
Thorpe SR, Baynes JW (2003) Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids 25:275–281
Uribarri J, Woodruff S, Goodman S et al (2010) Advanced glycation end products in foods and a practical guide to their reduction in the diet. J Am Diet Assoc 110:911–16.e12
Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL (2001) Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab 280:E685–E694
Weisenberger J (2014) Foods high in AGEs. http://www.diabetesforecastorg/2014/11-nov/foods-high-inages.html. Accessed 2014
Wendt TM, Bucciarelli LG, Lu X et al (2000) Accelerated atherosclerosis and vascular inflammation develop in apo-E null mice with type 2 diabetes. Circulation 102(Suppl):II-231
Willam C, Schindler R, Frei U, Eckardt KU (1999) Increases in oxygen tension stimulate expression of ICAM-1 and VCAM-1 on human endothelial cells. Am J Physiol 276:H2044–H2052
Wolf YG, Rasmussen LM, Ruoslahti E (1994) Antibodies against transforming growth factor-β 1 suppress intimal hyperplasia in a rat model. J Clin Invest 93:1172–1178
World Health Organization (2002) The World Health Report 2002. Reducing risks, promoting healthy life. World Health Organization, Geneva

Xu B, Chibber R, Ruggiero D, Kohner E, Ritter J, Ferro A (2003) Impairment of vascular endothelial nitric oxide synthase activity by advanced glycation end products. FASEB J 17: 1289–1291

Yamagishi S, Inagaki Y, Okamoto T et al (2002) Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J Biol Chem 277:20309–20315

Yang D, Elner SG, Bian ZM, Till GO, Petty HR, Elner VM (2007) Proinflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. Exp Eye Res 85:462–472

Yang X et al (2017) Oxidative stress-mediated atherosclerosis: mechanisms and therapies. Front Physiol 8:600

Yonekura H, Yamamoto Y, Sakurai S, Petrova RG, Abedin MJ, Li H (2003) Novel splice variants of the receptor for advanced glycation end products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. Biochem J 370: 1097–1109

Zhou Z, Wang K, Penn MS et al (2003) Receptor for AGE (RAGE) mediates neointimal formation in response to arterial injury. Circulation 107:2238–2243

Stem Cells in Dermatology: What the Future May Hold

2

Vinod Kumar and Sanjeev Handa

Abstract

Stem cells are unspecialized cells potentially used to repair and restore lost or defective function of tissues, and are increasingly being recognized as a futuristic therapy. Their differentiation potential ranges from pluripotent to unipotent. Skin has a diversified pool of stem cells that regularly repair or regenerate skin. The pathophysiology of skin repair and regeneration has immensely increased the interest of clinicians and researchers in skin stem cells. Dermal mesenchymal stem cells have been recognized as the source for replenishing dermal fibroblasts. Skin injury response stimulates the epidermal stem cells and follicular stem cells for re-epithelization and healing. Melanocyte stem cells are crucial in maintaining skin and hair color, while sebaceous gland stem cells contribute towards maintaining the barrier function of the skin. Other than this, skin is also known as the homing site of hematopoietic stem cells, which essentially maintains the skin lymphocyte subtypes and contributes to skin immunity. The generation of induced pluripotent stem cells has created new hopes for patients with genetic diseases. Correcting the genetic variations that result in disease and restoration of the natural protein expression is an exciting part of stem cell research. Several promising clinical trials have been done to correct skin diseases using melanocytes, keratinocytes, and mesenchymal stem cells. Still, there is a need to understand the efficacy and safety of stem cell therapies based on racial or ethnic differences. At present, we lack long follow-up clinical trials for skin diseases. Integration with bioengineering has improved the working of stem cells by providing a tissue-specific base to grow. The use of such biomaterials has succeeded in developing stem cell scaffolds. The biocompatibility of such

V. Kumar · S. Handa (✉)
Department of Dermatology, Venereology and Leprology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_2

materials however must be increased. The future of bioengineering is directed at the construction of various tissues including the skin, by using stem cells. Potential treatments are still some years away from clinical use, but results so far are promising.

Keywords

Skin · Stem cells · Regeneration · Bioengineering

2.1 Introduction

It has been known for more than 100 years, even intuitively before that, that a fusion of two cells (germ cells; sperm and oocyte) creates the potential to begin the life of a new human being. Fertilization begins an irreversible, directional process that is not only separate but genetically distinct from parents. These grown cells, which are unspecialized, undifferentiated, and have the potential to develop into a new organism, are considered stem cells. It was in the year 1868 when the term "stem cell" was reflected in the work of Ernst Haeckel, a German biologist. Haeckel had described unicellular organisms as the ancestors of multicellular organisms by using the term "Stammzelle." In his book Anthropogenie, he referred the term stem cell to the fertilized egg that potentially gave rise to all the cells of the organism. But this term was popularized by Edmund B. Wilson's book "The cell in development and inheritance." In the early nineteenth century, stem cells were established as the cells with the potential of self-renewal, proliferation, and differentiation to specialized cell types (Fig. 2.1) (Ramalho-Santos and Willenbring 2007).

Totipotent stem cells: These are cells with the potential of developing a whole organism. These cells can develop any organ of the body and its essential cells, for example, embryonic stem cells, the cells isolated after the fertilization zygote, and morula stage.

Pluripotent stem cells: These are cells with limited differentiation potential compared to totipotent stem cells. These cells cannot develop into a fully functional

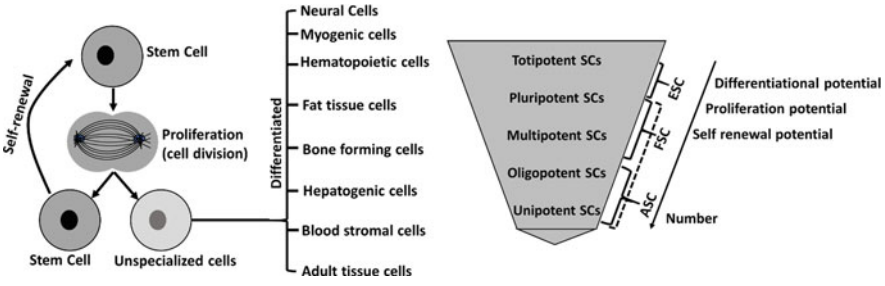


Fig. 2.1 Stem cell potency and their multilineage differentiation depending on their types. Characteristics (**a**) and types of stem cells (**b**)

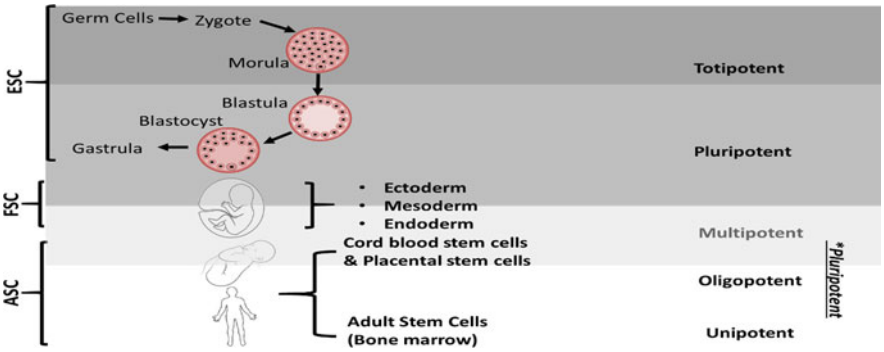


Fig. 2.2 Stem cells and their lineage differentiation. Stages leading to the formation into adult stem cells

organism, because of their inability to form the extraembryonic tissue like the placenta. Cells isolated at the stages after blastula are of this category.

Multipotent stem cells: These are cells with limited differentiation potential compared to pluripotent stem cells. These cells can differentiate and develop only in specialized defined germ layer cells. These include the cells for the ectoderm, mesoderm, and endoderm. Cells of the ectoderm cannot differentiate into the cells or organs of the mesoderm or endoderm.

Oligopotent stem cells: The differentiation potential of these cells is limited only to specialized cell types in a lineage. These include the cells of the connective tissue, nervous tissue, muscle tissue, and epithelial tissue.

Unipotent stem cells: These cells divide and differentiate only into one type of cells. This differentiation is meant to increase their cell number. These stem cells are known to reside within an organ or tissue. These include the cells of the heart (cardiac stem cells), liver (hepatic stem cells), kidney (renal stem cells), etc.

Based on the source of isolation, stem cells can be characterized into embryonic stem cell (ESC), foetus stem cell (FSC), and ASC (adult stem cell) (Diez Villanueva et al. 2012; Jiang et al. 2002; Pittenger et al. 1999; Verfaillie et al. 2002). The FSC is also a category of ASC (Fig. 2.2).

The multilineage differentiation of stem cells decrease from totipotent to unipotent cells. The localization of these cells also changes with individual development. These stem cells are found localized to each tissue or organ of an individual and are known as adult stem cells. They are less in number and require laborious methods to isolate. These ASCs hold all the stem cell properties of self-renewal, proliferation, and differentiation (Diez Villanueva et al. 2012). The most common difference between ESC and ASC are tabulated as follows.

Characters	ESC	ASC
Source of isolation	Early stages of embryo	All the adult tissues
Potency	Totipotent/pluripotent	Multipotent
Immune rejection	Yes	Less likely (except allogeneic)
Teratoma	High risk	Relatively low risk
Ethical and legal status	Highly involved	Relatively low
Chromosome length	No shortening	Shortening with aging
Telomerase levels	High	Low

Other than these naturally existing stem cells, a subset of cells called stem cell-like cells can be created in the laboratory. These include induced pluripotent stem cell (iPSC) and somatic cell nuclear transfer (SCNT). Both these cell types behave and possess all the properties of stem cells.

iPSC is a somatic cell that is genetically reprogrammed to the ESC-like cell stage through the forced expression of a set of genes. It was in the year 2006 when Yamanaka lab at the Kyoto University converted the mouse fibroblast cells to ESC-like cells. Later in 2007, Yamanaka and Thomson's group reprogrammed the human fibroblast cells (Takahashi and Yamanaka 2013). In the year 1962, Gurdon (1962), in his work on SCNT, claimed that the identity of a cell is reversible. Gurdon replaced the immature frog egg nucleus with the mature nucleus of interstitial cell. This fused cell developed into a normal tadpole. For this discovery, both John B. Gurdon and Shinya Yamanaka shared the 2012 Nobel Prize for medicine.

In the recent development of stem cell research, there are few confusing terms like progenitor and precursor cells (Fig. 2.3). Both these terms have been defining an ancestral cell interchangeably. Based on their different chromatin/epigenetic signature, the term progenitor or precursor can be used. A tissue can have more than one progenitor or precursor cells that differentiate and in combination form the tissue. But the tissue stem cell should be one. For example, during hematopoiesis, there is

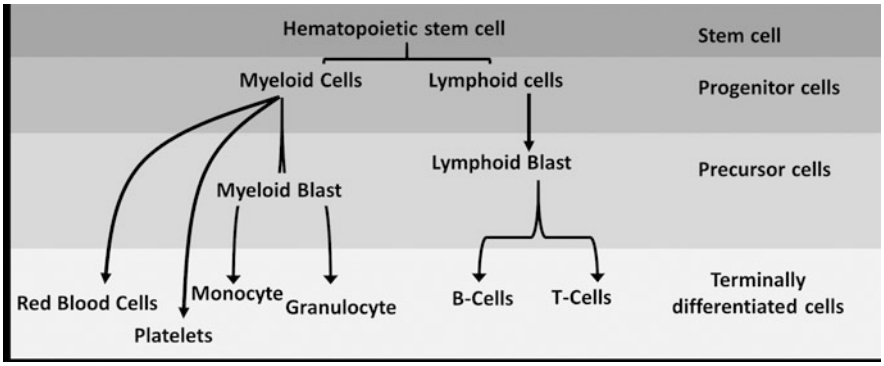


Fig. 2.3 Hematopoietic stem cell and their hierarchy of differentiation. Stages in the differentiation of stem cell into terminally differentiated cells

one hematopoietic stem cell and a set of progenitor and precursor cells that differentiate to terminally differentiated cells (Zakrzewski et al. 2019).

2.2 Skin Stem Cells

Cells of skin that help in its repair, regeneration, and maintaining skin integrity are known as skin stem cells. A variety of skin stem cells and progenitor cells have been described in the literature, due to skin cellular heterogeneity. Characterization and identification of these stem cells are tough and confusing due to their common or sharing microniche. The skin stem cells are categorized as ASCs. The diversity of skin stem cells is one of the largest known to date (Chu et al. 2018). These stem cells play an important role in maintaining the skin homeostasis, fast recovery, and repair, because of the vulnerability of skin to environmental stressors, ultraviolet radiation, trauma, and chemical burns and cracks. Abnormal elastin, collagen synthesis, decreased melanocyte, and keratinocyte number are associated consequences of these exposures. Cumulatively this leads to skin aging and deformation. The loss of functional integrity of the skin, especially the epidermis, decreases cutaneous water content, electrolytes, and increased skin or systemic infections. There are several inherited or acquired dermatological disorders responsible for this loss of skin integrity. Certain diverse events like burns, trauma, and drug reactions are also associated with skin deformation, which is taken care of by skin stem cells (Chu et al. 2018; Taub and Pham 2018).

2.3 Skin Stem Cell Niche

The stem cells localize to their specific space in the skin, known as a niche (Fig. 2.4). Till now there are five stem cell niches that have been identified in the skin including the basal layer, sebaceous gland, hair follicle bulge, dermal papilla, and dermis (Hsu et al. 2014). The basal layer is a house for 1–7% of the stem cell-like population, which can be identified with the expression of α6 and β1 integrins. The sebaceous gland is also identified as a niche for stem cells, which are expected as they descend from follicle stem cells. These stem cells are expressing Krt15, Lgr6, and Blump1 (Jaks et al. 2008; Nowak et al. 2008). These cells differentiate into sebocytes that degenerate to lubricate the skin by releasing sebum into the hair canal. Hair follicular niche is known to home the cells responsible for hair growth and generation. These stem cells localize at the base of the hair follicle and beneath the sebaceous gland under the hair lining. These cells can be identified with the expression of CD200, Krt15, and Lgr5 (Jaks et al. 2008; Nowak et al. 2008; Shpichka et al. 2019). The dermal papilla niche contains the stem cells originating from the neural crest during embryogenesis. This niche is proposed to be in coordination with follicular stem cells and send a signal to start the new anagen phase of hair growth. The dermal niche comprises the progenitor cells for melanocyte, fibroblast, and mesenchymal

Fig. 2.4 Organization of various layers of skin and skin stem cell niche

stem cells. These stem cells differentiate into skin myofibroblasts, melanocytes, and dermal mesenchyme to maintain skin integrity and tone.

Traditional or translational medicine has been adopted to restore or recover the skin integrity. Cell-based therapies hold great promise in repairing the defective epithelium including even the deeper skin layers. Skin stem cells which are identified to date are as follows:

1. **Epidermal stem cells (EpSCs):** The stem cells of the basal layer are called epidermal stem cells. These cells divide to maintain epidermis homeostasis and prompt wound healing or skin repair. The cell surface proteins used to identify these cells are p63, integrins$\beta 1^{high}$/MCSP^{+} (melanoma chondroitin sulfate proteoglycan), and integrins$\alpha 6^{high}$/CD71^{dim}. p63 is a member of p53 transcription factor family and known to regulate the proliferation of epidermal stem cells by controlling the expression of Notch, Wnt, and TGF-β cascades (Jones and Watt 1993; Ouji et al. 2008; Yang et al. 2020).
2. **Melanocyte stem cells:** Stem cells localized at dermal and bulge region belong to melanocyte stem cells. They are meant to replenish the existing skin melanocyte content. The cell markers used for the identification of these cells include Dct, Sox, and paired box protein 3. Paired box protein 3 crucially regulates the development of neural crest during embryogenesis. It further controls the expression of melanogenesis-associated gene like tyrosinase, tyrosinase-related protein (TRP-1), and dopachrome tautomerase (DCT), through the microphthalmia-associated transcription factor. Dct expression is involved in controlling the pigment color (Harris et al. 2013; Lang et al. 2005; Nishimura et al. 2002; Osawa et al. 2005) (Fig. 2.5).
3. **Hair Follicular stem cells (HFSCs):** Hair is a dynamic mini-organ that regenerates continuously throughout the life of an individual. Cells contributing to maintaining this continuous hair growth, regeneration, and localized to the follicular region are categorized as hair follicular stem cells. These bulge region

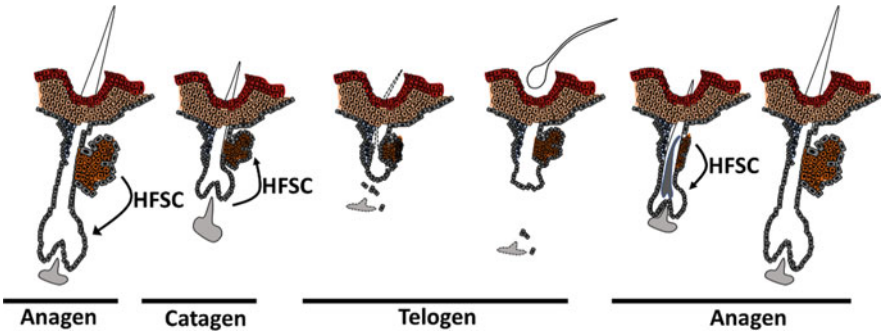


Fig. 2.5 Stem cells and hair cycle

cells express surface proteins keratin15, CD34, leucine-rich repeat-containing G-protein-coupled receptor 5, SRY-box transcription factor 9, LIM/homeobox protein Lhx2, nuclear factor of activated T-cells, cytoplasmic 1, pleckstrin homology-like domain family A member 1, and keratin19. These cells can be differentiated to hair follicle-epithelium, outer and inner root sheath, and hair shaft. Hair undergoes a three-step growth cycle: growth phase (anagen), regression (catagen), and rest (telogen). In the anagen phase, the HFSCs proliferate and contribute to the growth of the hair shaft and elongation of the hair follicle. Hair regression starts at the catagen stage and is completed in the telogen phase with no hair growth and shortened hair follicle. The HFSCs move from the basal region to the follicle bulge and reside there in a quiescent state till they receive a further stimulus to grow (Kloepper et al. 2008; Schneider et al. 2009; Stenn and Paus 2001).

4. **Sebaceous gland stem cells (SeSCs):** The holocrine gland attached to the hair follicle is a reservoir for Blimp1-marked stem cells. These stem cells differentiate and replenish the sebocytes, which are committed to continuous sebum production. With increasing age, despite a substantial sebaceous gland number, there is considerably less sebum secretion. That results in acute dryness and skin scalping. This might be attributed to the exhaustion of SeSCs. This leads us to understand the importance of localized stem cells. Apart from sebum, they were reported to have a significant role in wound healing and repair (Horsley et al. 2006; Ito et al. 2005).
5. **Dermis-mesenchymal stem cell-like cells (DMSCs):** A mesenchymal stem cell-like cell population has been identified in the dermis known as DMSCs. The precise anatomical localization of the DMSCs is entirely unclear. These cells are adherent and express mesenchymal stem cell-specific markers, i.e., CD70, CD90, and CD105. These are the derivatives of nonadherent dermal cells and display the capacity to differentiate into mesodermal or neural cell types. DMSCs have been shown to preserve the extracellular matrix of the skin and protect the skin microenvironment (Bartsch et al. 2005; Toma et al. 2005; Vaculik et al. 2012).

6. **Skin neural progenitor cells (skin-NPCs):** A distinct cell population that behaves like the neural crest stem cell has been identified in the skin and characterized as skin-NPCs. These cells exhibit the potential of neural cell differentiation and are believed to migrate into skin tissue during embryogenesis. Skin-NPCs have been shown to differentiate into smooth muscle, adipocyte, neuronal, glial, and osteoblast-like cells (Fernandes et al. 2004, 2008).
7. **Skin-hematopoietic stem cell-like cells (SHSCs):** The cells of dermal follicle papillae expressing the characteristic markers of hematopoietic stem cells are called SHSCs. These cells might divide into erythroid and myeloid lineages. These perivascular cells express CD34 and are predominantly localized in the vicinity of hair follicles. SHSCs can be isolated based on their collagen VI adhesion ability. They are presumed to be originated during embryogenesis and localized to skin tissue. Here these cells proliferate, differentiate, and neovascularize to maintain skin haemostasis (Fu and Sun 2009).

2.4 Skin Stem Cells and Their Localization

Stem cells	Location (niche)	Markers
Epidermal stem cells	Epidermal basal layer	p63, α6high/CD71dim, β1high/melanoma chondroitin sulfate proteoglycan + (MCSP+), (Jones and Watt 1993; Ouji et al. 2008; Yang et al. 2020)
Melanocyte stem cells	Hair follicle bulge region and hair germ	Dct, Sox, Pax3 (Harris et al. 2013; Lang et al. 2005; Nishimura et al. 2002; Osawa et al. 2005)
Hair follicle stem cells	Hair germ at base of hair follicle	NFIB, Lgr5, Sox9, Lhx2, K19, K15, PHLDA1, CD34,CD200, NFATC1, bromodeoxyuridine dye retention (Kloepper et al. 2008; Schneider et al. 2009; Stenn and Paus 2001)
Sebaceous gland stem cells	Sebaceous glands, infundibulum	Blimp1 (Horsley et al. 2006).(Ito et al. 2005)
Dermis-mesenchymal stem cell-like cells (DMSCs)	Dermis	CD70, CD105, CD90 (Bartsch et al. 2005; Toma et al. 2005; Vaculik et al. 2012)
Skin neural progenitor cells (skin-NPCs)	Bulge region	Nestin (Fernandes et al. 2004, 2008)
Skin-hematopoietic stem cell-like cells	Dermis	CD34 (Fu and Sun 2009)

2.5 Stem Cells and Skin Regeneration

Skin stem cells and skin regeneration: Skin regeneration is a cumulative contribution of both epidermal and follicular stem cells. The EpSCs contribute extensively to the repair and reepithelialization of wound tissue. Stressed external or internal elements and conditions instigate the EpSCs to rapidly divide and generate short-lived cells to carry out skin repair and regeneration. Usually, these stem cells remain quiescent but after a trigger they divide quickly and produce several cells (Fuchs 2008). Other than the EpSCs, stem cells from the bulge region of hair follicle (HFSC) and sebaceous gland (SeSC) also contribute substantially to skin rejuvenation. Skin injury reactivates these quiescent stem cells, prompt their migration to the site of injury, and increase participation in the regeneration process (Taylor et al. 2000). However, the HFSC and SeSC contribution to skin healing is transient. An interesting scientific observation has noted that HFSCs and SeSCs both participate considerably during acute wound healing in the beginning but later the HFSCs are replaced by EpSCs (Langton et al. 2008).This finding highlighted the fact that when the skin needs urgent stem cells, all the residing skin stem cells contribute, but they are not essentially required for the long-term maintenance of the skin. Skin repair happens basically in four phases: hemostasis (coagulation), inflammation (mononuclear cell infiltration), proliferation (granulation and angiogenesis), and maturation (scar formation with collagen deposits). Keratinocytes and fibroblasts are the primary regenerating cells of healing or regenerating the skin. Keratinocytes contribute to the formation of tight cellular junctions as well as stratified structure formation. Whereas fibroblasts produce extracellular matrix components and secrete essential growth factors or cytokines required for skin repair, a new stratified epidermis is reestablished along with basal lamina once the wound surface gets covered with a keratinocyte monolayer (Zhang and Fu 2008).The Wnt/b-catenin signalling pathway is activated during skin repair. The high Wnt levels induce stem cells to differentiate and develop hair structures and sebaceous glands while low levels are associated with their epithelial differentiation. Another signalling pathway is the Notch signalling cascade. The skin stem cells have high expression of Notch1, which directs the differentiation of stem cell to epithelial cells and increases cellular adhesion (Ouji et al. 2008). The follicular stem cells divide and migrate to restore the barrier function. The HFSC releases contact inhibition after the injury and migrates to the leading edge parallel with keratinocytes. Despite the significant involvement of HFSCs or SeSCs in the repair process, the regeneration of hair follicles and sweat glands is challenging (Li et al. 2019). It has been reported that the EpSCs can differentiate and give rise to nascent hair follicles outside the hair follicle stem cell niche, suggesting that the immature EpSCs have the potential to replenish hair follicles when in need. On the other hand, Fe et al. showed differentiation of the EpSCs to sweat gland cells. Revascularization is an important event needed for the quick recovery of the damaged skin. Hypoxia-inducible factor 1, VEGF, and CXCL12 are known to initiate angiogenesis in damaged skin. They are believed to increase the import of non-skin stem cells to the site of repair. The skin stem cells are also responsible for the intracutaneous growth factor production which accelerates

the repair process. Fibroblast growth factor (FGF) increases the recruitment of MSCs and keratinocyte migration to fill the wounded area. An increased stem cell growth can be achieved with the FGF expression. The major signalling pathways associated with skin stem cells are as follows (Blanpain and Fuchs 2006):

In situ location	Epidermal stem cell	Dermal stem cell
Signalling pathways	Wnt, β-catenin, Shh, TGFβ, BMPs, p63	Wnt, Timp, BMPs, FGF, Shh, IGF, Notch
Surface and structural proteins	K5, K14, K15, E-cadherin, LGR5, LGR6, CD29, CD34, CD49f, CD117, CD200	NG2, CD34, CD44, CD54, CD73, CD90, CD105, CD133, CD271
Potential matrix components	Laminin fibrin Collagen IV collagen I Collagen XVII Nephronectin	

Non-Skin stem cells and skin regeneration: Stem cells other than those from the skin are equally participating in skin regeneration. **Mesenchymal stem cells (MSCs)** are localized in bone marrow and migrate to the site of injury to differentiate and regulate tissue regeneration. The regenerative potential of the MSCs is derived by their secreted growth factor, cytokines, chemokines (Burdon et al. 2011). These cells can be isolated and infused in the damaged skin area to achieve fast recovery. Their hypoimmunogenicity and capacity to release the immunomodulatory cytokines make them best suited for clinical infusions, besides the absence or minimal expression of MHC class II and co-stimulatory molecules like CD40, CD40 ligand and CD80 and CD86. Other than bone marrow, the tissues like adipose tissue, placenta, dental pulp, umbilical cord, cord blood, etc. can be exploited as the potential MSC extracting sources. MSCs can differentiate into fibroblast-like cells to support skin regeneration. MSCs have been described to be used in chronic wound healing by Badiavas and Falanga (2003). This resulted in the recruitment of inflammatory cells, increased reticulin fibres, vascularity, and cellularity of the wound which was absent before the MSCs treatment. By this treatment, the authors achieved healing of wounds that were not responding for a year. Later *Falanga* et al. injected MSCs to promote elastin production and a reduction in the wound size (Falanga et al. 2007). *Dash* et al. studied infusion of MSCs in 24 patients with nonhealing ulcers (diabetic ulcers and Buerger's disease) to achieve reduction in ulcer size, increase pain free movement, and promote fibroblast cells within the wound (Dash et al. 2009) (Fig. 2.6).

Hematopoietic stem cells (HSCs) include CD34 and CD45 expressing cells with the capacity to differentiate and replenish blood cells are hematopoietic stem cells. The regenerative or healing potential of these cells is thought to be delivered by their immunomodulatory ability. They have been shown to produce epithelial and hepatic cells (Sackstein 2004).These cells express CD44 which binds to E-selectin and results in strong homing to the inflammatory site. In the year 2014, *Wettestein* et al. has tested the effect of HSCs on pressure sores and reported a decrease in the wound size with no sign of malignancy (Wettstein et al. 2014).

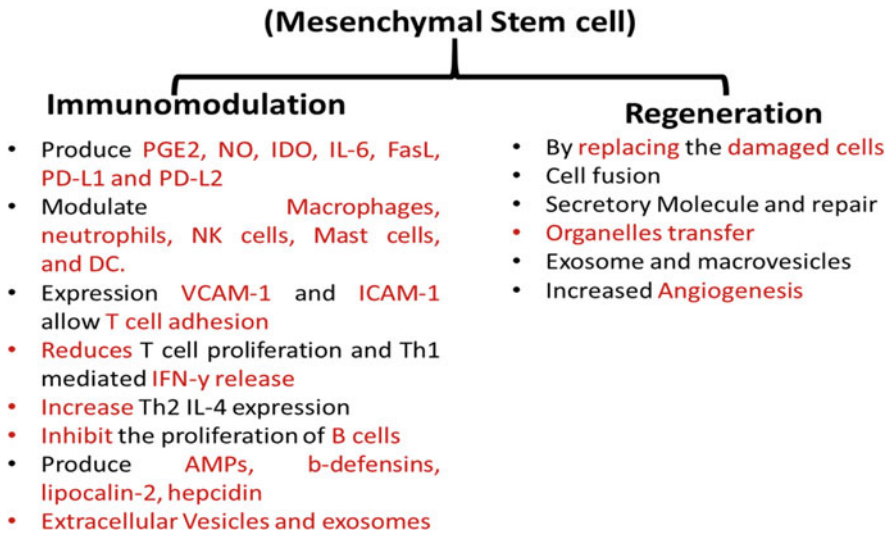


Fig. 2.6 Mesenchymal stem cells

Endothelial progenitor cells (EPCs) include CD34, CD133, and VEGFR-2-expressing cells which reside in the peripheral tissue and bone marrow (Roncalli et al. 2008). Reduced blood flow, stroke, and ischemia are the events which trigger EPCs migration to initiate revascularization and tissue repair. The bone marrow-migrated EPCs incorporate into the growing vasculature of ischemic regions (Rosell et al. 2013). Their migration is accomplished by endothelial NOs, NO, VEGF-A, MM-9, and GM-CSF. The secreted angiogenic factors like PDGF, VEGF, macrophage inflammatory protein, and FGF-2 also help in EPCs mobilization (Barcelos et al. 2009; Suh et al. 2005; Zhang and Chopp 2013).

2.6 Stem Cell Therapy and Dermatological Disease

Epidermolysis bullosa: It is a hereditary skin blistering disease with clinical and genetic heterogenicity, but all the forms present with mechanically induced skin fragility and blistering. Based on the morphological presentation of the blisters, EB is categorized into four types: EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB), and Kindler syndrome (KS). The blister is localized in the epidermis of EBS, along the basement membrane in JEB, below the basement membrane in DEB, and disorganized in the epidermal-dermal junction in Kindler syndrome (Has et al. 2020). To assess the potential of cellular therapy in correcting the EB pathophenotype, *Wong* et al. gave a single allogeneic intradermal injection of normal human fibroblast cells. There was an increase in the collagen VII expression and anchoring fibrils, although the morphology was not normal, because of the increased accumulation of mutated protein (Wong et al. 2008). This study also revealed that the treatment modality was painful, difficult, and intolerable. Similar results were

also reported in another study of allogeneic fibroblast transplants (Bruckner-Tuderman 2019). This limited success prompted the systemic injection of stem cells to correct the EB phenotype. But systemic allogeneic stem cell therapies failed to cure DEB and resulted in adverse events including the death of the recipient patients (Hammersen et al. 2016; Rashidghamat and McGrath 2017; Venugopal et al. 2013). In the year 2010, allogeneic MSCs were used for the first time in 13- and 25-year-old RDEB patients through intradermal injection. Both the patients had increased collagen expression and healing (Wagner et al. 2010). Subsequently, with modifications, MSCs were injected through intravenous route and displayed increased healing rate and decreased new blister numbers. These intravenous injections improved the disease but there was no increase in the collagen expression. At present, the number of patients treated with cellular therapies is exceptionally low and is not equally effective in all the recipients. Therefore, the establishment of such therapies for EB patients still needs more aggressive design and monitoring. Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Biological: mesenchymal stem cells derived from bone marrow (BM-MSCs)	Safety Study and Preliminary Efficacy of Infusion Haploidentical Mesenchymal Stem Cells Derived from Bone Marrow for Treating Recessive Dystrophic Epidermolysis Bullosa	9	Active, not recruiting	Hospital Universitario La Paz Madrid, Spain
Genetically corrected cultured epidermal autograft (ATMP)	Clinical Trial to Assess Safety and Efficacy of Autologous Cultured Epidermal Grafts Containing Epidermal Stem Cells Genetically Modified in Patients With JEB	1	Active, not recruiting	EB House Austria, Department of Dermatology, Paracelsus Medical University Salzburg, Austria
Allogeneic hematopoietic stem cell	Allogeneic Hematopoietic Stem Cell Transplant For Epidermolysis Bullosa	7	Terminated	Masonic Cancer Center, University of Minnesota Minneapolis, Minnesota, United States
Allogeneic stem cell	Allogeneic ABCB5-positive Stem Cells for Treatment of Epidermolysis Bullosa	16	Active, not recruiting	University of Minnesota, Masonic Cancer Center and Medical Center Minneapolis, Minnesota, United States

Vitiligo: A significant destruction of skin pigment cells driven by autoimmunity, genetics, oxidative stress, and environmental components leads to depigmentation of the skin. The exact pathogenesis of vitiligo is still unknown. Transplantation of in vitro cultured cells like keratinocyte and melanocyte to the depigmented area results in gaining of skin color (Pandya et al. 2005). Transplantation of cultured melanocytes is an expensive procedure and needs expertise and established culturing facility. It was in the year 1992 when Gauthier and Surleve-Bazeille used non-cultured epidermal suspension (NCES) technique for treating stable vitiligo (Gauthier and Surleve-Bazeille 1992). The available data suggests that there is no statistical difference in the rate of repigmentation with the infusion of cultured or non-cultured melanocytes (Gill et al. 2019). Researchers have used a dermabrader to scrape the white skin patches and sprayed them with a suspension of skin cells. Gradually, the sprayed skin cells including the melanocytes start growing and repigmenting the skin area (Hamza et al. 2019). On average about 45% of the skin returned to its original color. However, the result varies in individual patients. This therapy works well in focal or segmental vitiligo patients because the disease is very stable and affects only one part of the body. In such patients, the maximum repigmentation observed has been up to 68%. However, patients with symmetrical vitiligo did not do as well from such therapies, due to their more active immune system. Even in stable vitiligo, the extent of repigmentation depends on the location of the white patch. The vitiligo lesions on the legs, feet, face, and forearms respond favorably to this therapy while poor response is seen on elbows and acral areas of the hand (Zokaei et al. 2019). Hair follicle outer root sheath cell suspension is also being also used to treat stable vitiligo skin. Here the stem cells of the hair sheath have been prepared by digesting the hair follicle with trypsin-EDTA. This suspension has been reported as being equally effective as NCES (Thakur et al. 2019). Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Melanocyte keratinocyte transplantation	Evaluating the Efficacy of the Melanocyte Keratinocyte Transplantation Procedure in the Treatment of Vitiligo	17	Recruiting	University of California, Irvine Irvine, California, United States
Melanocyte transplantation	Autologous Transplantation of Melanocytes for Treatment of Vitiligo Skin	300	Completed	Johns Hopkins Outpatient Center Baltimore, Maryland, United States
Grafting with epidermal cells	Epidermal Cell Transplantation in Vitiligo Skin	23	Completed	Centre hospitalier Universitaire de Nice Nice, France

Alopecia: Hair loss occurs quite commonly in men and women. There are only two drugs approved by the FDA for its treatment, minoxidil and finasteride (Egger et al. 2020). However, both drugs are associated with several side effects and have a low efficacy. In pattern hair loss, the HFSCs remain viable and make it a reversible condition. Novel treatment modalities need to utilize or activate these cells by providing adequate signals and environment. Stem cell transplant, secretome, and exosomes are the new options used to activate the HFSCs (Falto-Aizpurua et al. 2014; Erratum 2015). Bone marrow-derived mononuclear cells and follicular stem cells of unaffected scalp transplanted in patients with alopecia areata (AA) and androgenetic alopecia (AGA) are able to improve the hair growth significantly without any adverse events. The use of HFSCs can increase the hair density of AGA patients. MSC (adipose tissue) infusion in patients with AA has improved hair density and growth. Other than these dermal papilla stem cells, primary pluripotent stem cells have been tested with favorable results, with an increase in hair density. Now the focus is to use the stem-cell secreted potential hair growth bioactive molecules including extra vesicle, nucleic acids, and proteins collectively called secretome (Elmaadawi et al. 2018). Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Stem cell educator therapy combined with oral minoxidil	Clinical Application of Stem Cell Educator Therapy in Alopecia Areata	20	Not yet recruiting	Yale University School of Medicine New Haven, Connecticut, United States
Adipose derived stem cells, PRP	Adipose Derived Stem Cells Versus Platelet Rich Plasma on Follicular Unit Extraction	40	Recruiting	Assiut University Hospitals, Assiut, Egypt
Adipose derived stem/stromal cells, PRP	AGA Biocellular Stem/ Stromal Hair Regenerative Study (STRAAND)	60	Recruiting	Irvine, California, United States

Atopic dermatitis (AD): It is a chronic inflammatory disorder presenting with eczematous cutaneous lesions and severe pruritus. Stem cells, preferably the MSCs, may be found suitable for the treatment of AD, because of their immunomodulatory properties. MSC infusion has been linked to reduce or slow down the allergic progression of AD, irrespective of their sources of isolation, i.e., bone marrow, adipose tissue, and umbilical cord blood. MSCs inhibited the T- and B-cell proliferation and increased the production of anti-inflammatory cytokines (IL-10 and TGF-β). Subcutaneous injection of human umbilical cord blood-derived MSCs in the eczematous area is able to reduce the Eczema Area and Severity Index (EASI) scoring without any side effects (Kim et al. 2017). Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Adult MSCs (FURESTEM-AD)	Safety and Efficacy of FURESTEM-AD Inj. in Patients with Moderate to Severe Chronic Atopic Dermatitis (AD)	197	Unknown	Kang Stem Biotech Co., Ltd. Korea
Stem cell bone marrow-acute graft versus host disease (SCM-AGH)	Safety and Efficacy of SCM-AGH in Subjects with Moderate to Severe Atopic Dermatitis	92	Recruiting	Inha University Hospital, Korea

Urticaria: It occurs in 0.5–5% of the general population. About, 30–40% of the patients are resistant to treatment and need new and effective modalities. There is only one study published in the year 2020, using autologous mesenchymal stem cells to treat chronic spontaneous urticaria. This study concluded that MSCs resulted in longer, more effective recovery and may be an alternative for treating resistant chronic spontaneous urticaria (Ozgul Ozdemir et al. 2020). Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Autologous mesenchymal stem cell	Experimental Autologous Mesenchymal Stem Cell Therapy in Treatment of Chronic Autoimmune Urticaria	10	Completed	Celal Bayar University, Medical School Manisa, Turkey

Scleroderma: Scleroderma is an autoimmune disease affecting the skin and other organs in which the skin is thick, hard, and scarred along with damage to the internal organs. Stem cells are well described for their immunomodulatory effect and have been used to treat scleroderma. HSCs transplantation improved the skin sclerosis and stabilized the pulmonary function. Myeloablative autologous stem cell transplantation successfully achieved long-term benefits in patients with scleroderma (Sullivan et al. 2018). Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Allogeneic mesenchymal stem cell	Treatment of Refractory Sever Systemic Scleroderma by Injection of Allogeneic Mesenchymal Stem Cells	20	Active, not recruiting	Saint-Louis Hospital Paris, France
Allogeneic hematopoietic stem cell transplantation	TBI Using IMRT and Cyclophosphamide Prior to Stem Cell Transplant for the Treatment of Severe Systemic Sclerosis	15	Recruiting	City of Hope Medical Center Duarte, California, United States

(continued)

Type of stem cells	Study	No. of patients	Status	Country
Autologous hematopoietic stem cell transplantation	Scleroderma Treatment with Autologous Transplant (STAT) Study	20	Active, not recruiting	City of Hope Comprehensive Cancer Center Duarte, California, United States
Autologous adipose tissue stem cell	Subcutaneous Injections of Autologous ASC to Heal Digital Ulcers in Patients with Scleroderma	32	Recruiting	Grenoble Hospital Grenoble, France

Psoriasis: Psoriasis is driven by a dysregulated immune system, with enhanced keratinocyte proliferation. The immune cell infiltration and creation of proinflammatory niche essentially stimulate these proliferative events. Lowering the inflammation is likely to help in reversing the disease and associated pathophenotypes. The infusion of stem cells has been reported to be effective and curative for severe psoriasis patients without long-term reoccurrence (Ciurea et al. 2019). These listed clinical trials are exploiting the role of stem cells in treating patients with psoriasis. Table (ClinicalTrials.gov).

Type of stem cells	Study	No. of patients	Status	Country
Umbilical cord-derived mesenchymal stem cells	Clinical Research on Treatment of Psoriasis by Human Umbilical Cord-derived Mesenchymal Stem Cells	12	Recruiting	Xiangya Hospital, Central South University Changsha, Hunan, China
Adipose-derived multipotent mesenchymal stem cells	Safety and Efficacy of Expanded Allogeneic AD-MSCs in Patients with Moderate to Severe Psoriasis	7	Active, not recruiting	Guangdong Provincial Hospital of Traditional Chinese Medicine Guangzhou, Guangdong, China
Adipose-derived multipotent mesenchymal stem cells	Efficacy and Safety of AD-MSCs Plus Calpocitriol Ointment and PSORI-CM01 Granule in Psoriasis Patients	8	Recruiting	Guangdong Provincial Hospital of Traditional Chinese Medicine Guangzhou, Guangdong, China
Mesenchymal stem cells	Safety of FURESTEM-CD Inj. in Patients with Moderate to Severe Plaque-type Psoriasis	9	Recruiting	The Catholic Univ. Korea Seoul, St. Marry's Hospital Seoul, Korea

2.7 Future of Stem Cells in Skin Disease

Despite recent advancements in the treatment of skin diseases and its bioengineering, perfect regeneration is still a challenge. To what extent the skin stem cells can contribute to the regeneration of scar-free skin is uncertain. Non-skin stem cells are unspecialized cells which differentiate and replace damaged or nonfunctional cells. Their contribution to skin repair and regeneration is well recognized. However, the observed relief lasts for a few weeks or months. No doubt the coming years will reveal the stem cell working molecular mechanism and new critical genes controlling their fate.

Efforts are needed to understand the stem cell working mechanisms in skin disease by establishing animal models. To find or develop a specified animal model is tough, because of their differences in skin architecture and properties. Mice are commonly used animal model to study skin diseases, but their skin is quite different from human skin. Mouse skin is thin and has a compromised global nucleotide excision repair system. The mouse melanocytes are localized to the dermis, while in humans they are in the basal layer. Moreover, their hair growth cycle is synchronized, while in humans it is independent of each other. The hair cycle is also short in mice and lasts only for 3 months, while in humans it lasts years.

The other challenge is to identify the authentic stem cell of skin. Due to the diverse cellular heterogenicity of skin cells, the identification of stem cells is a tough job. Within skin, five niches have been delineated, i.e., basal layer, hair follicle bulge, sebaceous gland base, dermal papillae, and dermis. Each niche harbours different stem cell populations, and exact identification of these cell types is essential in mapping their therapeutic use. LRIG1, FRMD4A, Lgr5, Sox9, Krt15, and Lgr6 are the few markers which are used to identify these cells. Still the potential of these cells alone or in combination is not enough to maintain skin integrity. Hence, there is a possibility of an as yet unidentified stem cell or other factors which essentially back skin regeneration during the life of an individual.

Somatic cell reprogrammed stem cells and induced pluripotent stem cells are a major breakthrough of the century. It helps in the development of customized, patient-specific cells. Dermal cell-specific differentiation of iPSCs of humans and mice has been achieved. A major issue in this technique of generating induced pluripotent stem cells is the untargeted cell genomic DNA modification, because of the genomic integrating viruses during reprogramming. Injection of therapeutic genes housing adenovirus vector has been seen to elicit a strong immune response and multi-organ failure leading to death. Another disadvantage associated with the cell reprogramming is unpredicted trans- or genomic gene silencing and activation. This is not only affecting cells' capacity to differentiate but also their commitment to a lineage. Such modifications can lead to the generation of teratoma. To overcome this flaw, it is a need to adopt genomic nonintegrating RNA virus, Sendai virus, to generate transgene-free iPSCs.

Populations with inherited skin diseases where the skin barrier and function are compromised, desperately need a therapy to restore an intact epithelium. Infusion of allogeneic cells is associated with the risk of rejection and mortality, while through

iPSCs we can generate genetically engineered patient-specific stem cells, which then can further be differentiated into keratocytes and fibroblasts. At present the genomic integration of reversal genes (oct, kfl4, sox2, and cmyc) of iPSCs is restricting their clinical use. So, the advancement in generating non-genome-integrating iPSCs using Sendai virus may be the key to future therapeutic applications.

CRISPER/Cas9-based genome editing is a new possibility that can be utilized in correcting the inherited skin diseases. In a recent study, Jackow et al. (2016) using this technique successfully edited the fibroblasts of dystrophic epidermolysis bullosa causing mutation of COL7A gene. The iPSCs generated after cas9 gene editing potentially differentiate and generate human skin equivalents to restore collagen VII expression. However, this technique also depends on adenovirus and does not avert the associated risk. The high off-target effects which are observed with CRISPR/Cas9 and induction of DNA damage toxicity is a major concern. In addition to this, a major portion of human subjects having antibodies to Cas9 (Uddin et al. 2020) and the unpredicted immune response toward adenovirus limit the wide use of this technique and definitely need further modifications or advancements.

References

Badiavas EV, Falanga V (2003) Treatment of chronic wounds with bone marrow-derived cells. Arch Dermatol 139(4):510–516. https://doi.org/10.1001/archderm.139.4.510

Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M, Simm A, Campagnolo P, Mangialardi G, Stevanato L, Alessandri G, Emanueli C, Madeddu P (2009) Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 104(9):1095–1102. https://doi.org/10.1161/CIRCRESAHA.108.192138

Bartsch G, Yoo JJ, De Coppi P, Siddiqui MM, Schuch G, Pohl HG, Fuhr J, Perin L, Soker S, Atala A (2005) Propagation, expansion, and multilineage differentiation of human somatic stem cells from dermal progenitors. Stem Cells Dev 14(3):337–348. https://doi.org/10.1089/scd.2005.14.337

Blanpain C, Fuchs E (2006) Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 22:339–373. https://doi.org/10.1146/annurev.cellbio.22.010305.104357

Bruckner-Tuderman L (2019) Newer treatment modalities in epidermolysis bullosa. Indian Dermatol Online J 10(3):244–250. https://doi.org/10.4103/idoj.IDOJ_287_18

Burdon TJ, Paul A, Noiseux N, Prakash S, Shum-Tim D (2011) Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. Bone Marrow Res 2011:207326. https://doi.org/10.1155/2011/207326

Chu GY, Chen YF, Chen HY, Chan MH, Gau CS, Weng SM (2018) Stem cell therapy on skin: mechanisms, recent advances and drug reviewing issues. J Food Drug Anal 26(1):14–20. https://doi.org/10.1016/j.jfda.2017.10.004

Ciurea SO, Hansrivijit P, Ciurea AM, Hymes S, Chen J, Rondon G, Hosing C, Popat U, Champlin RE (2019) Curative potential of hematopoietic stem cell transplantation for advanced psoriasis. Am J Hematol 94(6):E176–E180. https://doi.org/10.1002/ajh.25470

ClinicalTrials.gov. https://clinicaltrials.gov/ct2/results?cond=Vitiligo & term=stem+cell & cntry= & state= & city= & dist=

Dash NR, Dash SN, Routray P, Mohapatra S, Mohapatra PC (2009) Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. Rejuvenation Res 12(5):359–366. https://doi.org/10.1089/rej.2009.0872

Diez Villanueva P, Sanz-Ruiz R, Nunez Garcia A, Fernandez Santos ME, Sanchez PL, Fernandez-Aviles F (2012) Functional multipotency of stem cells: what do we need from them in the heart? Stem Cells Int 2012:817364. https://doi.org/10.1155/2012/817364

Egger A, Tomic-Canic M, Tosti A (2020) Advances in stem cell-based therapy for hair loss. CellR4 Repair Replace Regen Reprogram 8:e2894. https://www.ncbi.nlm.nih.gov/pubmed/32968692

Elmaadawi IH, Mohamed BM, Ibrahim ZAS, Abdou SM, El Attar YA, Youssef A, Shamloula MM, Taha A, Metwally HG, El Afandy MM, Salem ML (2018) Stem cell therapy as a novel therapeutic intervention for resistant cases of alopecia areata and androgenetic alopecia. J Dermatolog Treat 29(5):431–440. https://doi.org/10.1080/09546634.2016.1227419

Erratum (2015) Expert Opin Emerg Drugs 20(2):347. https://doi.org/10.1517/14728214.2015.1027860

Falanga V, Iwamoto S, Chartier M, Yufit T, Butmarc J, Kouttab N, Shrayer D, Carson P (2007) Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue Eng 13(6):1299–1312. https://doi.org/10.1089/ten.2006.0278

Falto-Aizpurua L, Choudhary S, Tosti A (2014) Emerging treatments in alopecia. Expert Opin Emerg Drugs 19(4):545–556. https://doi.org/10.1517/14728214.2014.974550

Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F, Biernaskie J, Junek A, Kobayashi NR, Toma JG, Kaplan DR, Labosky PA, Rafuse V, Hui CC, Miller FD (2004) A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6(11):1082–1093. https://doi.org/10.1038/ncb1181

Fernandes KJ, Toma JG, Miller FD (2008) Multipotent skin-derived precursors: adult neural crest-related precursors with therapeutic potential. Philos Trans R Soc Lond Ser B Biol Sci 363(1489):185–198. https://doi.org/10.1098/rstb.2006.2020

Fu X, Sun X (2009) Can hematopoietic stem cells be an alternative source for skin regeneration? Ageing Res Rev 8(3):244–249. https://doi.org/10.1016/j.arr.2009.02.002

Fuchs E (2008) Skin stem cells: rising to the surface. J Cell Biol 180(2):273–284. https://doi.org/10.1083/jcb.200708185

Gauthier Y, Surleve-Bazeille JE (1992) Autologous grafting with noncultured melanocytes: a simplified method for treatment of depigmented lesions. J Am Acad Dermatol 26(2 Pt 1):191–194. https://doi.org/10.1016/0190-9622(92)70024-a

Gill BS, Brar MS, Chaudhary N, Randhawa A (2019) Non-cultured melanocyte transfer in the management of stable vitiligo. J Family Med Prim Care 8(9):2912–2916. https://doi.org/10.4103/jfmpc.jfmpc_546_19

Gurdon JB (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 10:622–640. https://www.ncbi.nlm.nih.gov/pubmed/13951335

Hammersen J, Has C, Naumann-Bartsch N, Stachel D, Kiritsi D, Soder S, Tardieu M, Metzler M, Bruckner-Tuderman L, Schneider H (2016) Genotype, clinical course, and therapeutic decision making in 76 infants with severe generalized junctional epidermolysis bullosa. J Invest Dermatol 136(11):2150–2157. https://doi.org/10.1016/j.jid.2016.06.609

Hamza AM, Hussein TM, Shakshouk HAR (2019) Noncultured extracted hair follicle outer root sheath cell suspension versus noncultured epidermal cell suspension in the treatment of stable vitiligo. J Cutan Aesthet Surg 12(2):105–111. https://doi.org/10.4103/JCAS.JCAS_136_18

Harris ML, Buac K, Shakhova O, Hakami RM, Wegner M, Sommer L, Pavan WJ (2013) A dual role for SOX10 in the maintenance of the postnatal melanocyte lineage and the differentiation of melanocyte stem cell progenitors. PLoS Genet 9(7):e1003644. https://doi.org/10.1371/journal.pgen.1003644

Has C, Bauer JW, Bodemer C, Bolling MC, Bruckner-Tuderman L, Diem A, Fine JD, Heagerty A, Hovnanian A, Marinkovich MP, Martinez AE, McGrath JA, Moss C, Murrell DF, Palisson F, Schwieger-Briel A, Sprecher E, Tamai K, Uitto J, Woodley DT, Zambruno G, Mellerio JE (2020) Consensus reclassification of inherited epidermolysis bullosa and other disorders with skin fragility. Br J Dermatol 183(4):614–627. https://doi.org/10.1111/bjd.18921

Horsley V, O'Carroll D, Tooze R, Ohinata Y, Saitou M, Obukhanych T, Nussenzweig M, Tarakhovsky A, Fuchs E (2006) Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. Cell 126(3):597–609. https://doi.org/10.1016/j.cell.2006.06.048

Hsu YC, Li L, Fuchs E (2014) Emerging interactions between skin stem cells and their niches. Nat Med 20(8):847–856. https://doi.org/10.1038/nm.3643

Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, Cotsarelis G (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med 11(12):1351–1354. https://doi.org/10.1038/nm1328

Jackow J, Titeux M, Portier S, Charbonnier S, Ganier C, Gaucher S, Hovnanian A (2016) Gene-corrected fibroblast therapy for recessive dystrophic epidermolysis bullosa using a self-inactivating COL7A1 retroviral vector. J Invest Dermatol 136(7):1346–1354. https://doi.org/10.1016/j.jid.2016.02.811

Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H, Toftgard R (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40(11):1291–1299. https://doi.org/10.1038/ng.239

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49. https://doi.org/10.1038/nature00870

Jones PH, Watt FM (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73(4):713–724. https://doi.org/10.1016/0092-8674(93)90251-k

Kim HS, Lee JH, Roh KH, Jun HJ, Kang KS, Kim TY (2017) Clinical trial of human umbilical cord blood-derived stem cells for the treatment of moderate-to-severe atopic dermatitis: phase I/IIa studies. Stem Cells 35(1):248–255. https://doi.org/10.1002/stem.2401

Kloepper JE, Tiede S, Brinckmann J, Reinhardt DP, Meyer W, Faessler R, Paus R (2008) Immunophenotyping of the human bulge region: the quest to define useful in situ markers for human epithelial hair follicle stem cells and their niche. Exp Dermatol 17(7):592–609. https://doi.org/10.1111/j.1600-0625.2008.00720.x

Lang D, Lu MM, Huang L, Engleka KA, Zhang M, Chu EY, Lipner S, Skoultchi A, Millar SE, Epstein JA (2005) Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature 433(7028):884–887. https://doi.org/10.1038/nature03292

Langton AK, Herrick SE, Headon DJ (2008) An extended epidermal response heals cutaneous wounds in the absence of a hair follicle stem cell contribution. J Invest Dermatol 128(5):1311–1318. https://doi.org/10.1038/sj.jid.5701178

Li B, Hu W, Ma K, Zhang C, Fu X (2019) Are hair follicle stem cells promising candidates for wound healing? Expert Opin Biol Ther 19(2):119–128. https://doi.org/10.1080/14712598.2019.1559290

Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S (2002) Dominant role of the niche in melanocyte stem-cell fate determination. Nature 416(6883):854–860. https://doi.org/10.1038/416854a

Nowak JA, Polak L, Pasolli HA, Fuchs E (2008) Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell 3(1):33–43. https://doi.org/10.1016/j.stem.2008.05.009

Osawa M, Egawa G, Mak SS, Moriyama M, Freter R, Yonetani S, Beermann F, Nishikawa S (2005) Molecular characterization of melanocyte stem cells in their niche. Development 132(24):5589–5599. https://doi.org/10.1242/dev.02161

Ouji Y, Yoshikawa M, Moriya K, Nishiofuku M, Matsuda R, Ishizaka S (2008) Wnt-10b, uniquely among Wnts, promotes epithelial differentiation and shaft growth. Biochem Biophys Res Commun 367(2):299–304. https://doi.org/10.1016/j.bbrc.2007.12.091

Ozgul Ozdemir RB, Ozdemir AT, Kirmaz C, Ovali E, Olmez E, Kerem H, Evrenos MK, Deniz G (2020) Mesenchymal stem cells: a potential treatment approach for refractory chronic

spontaneous urticaria. Stem Cell Rev Rep 17(3):911–922. https://doi.org/10.1007/s12015-020-10059-w

Pandya V, Parmar KS, Shah BJ, Bilimoria FE (2005) A study of autologous melanocyte transfer in treatment of stable vitiligo. Indian J Dermatol Venereol Leprol 71(6):393–397. https://doi.org/10.4103/0378-6323.18942

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147. https://doi.org/10.1126/science.284.5411.143

Ramalho-Santos M, Willenbring H (2007) On the origin of the term "stem cell". Cell Stem Cell 1(1):35–38. https://doi.org/10.1016/j.stem.2007.05.013

Rashidghamat E, McGrath JA (2017) Novel and emerging therapies in the treatment of recessive dystrophic epidermolysis bullosa. Intractable Rare Dis Res 6(1):6–20. https://doi.org/10.5582/irdr.2017.01005

Roncalli JG, Tongers J, Renault MA, Losordo DW (2008) Endothelial progenitor cells in regenerative medicine and cancer: a decade of research. Trends Biotechnol 26(5):276–283. https://doi.org/10.1016/j.tibtech.2008.01.005

Rosell A, Morancho A, Navarro-Sobrino M, Martinez-Saez E, Hernandez-Guillamon M, Lope-Piedrafita S, Barcelo V, Borras F, Penalba A, Garcia-Bonilla L, Montaner J (2013) Factors secreted by endothelial progenitor cells enhance neurorepair responses after cerebral ischemia in mice. PLoS One 8(9):e73244. https://doi.org/10.1371/journal.pone.0073244

Sackstein R (2004) The bone marrow is akin to skin: HCELL and the biology of hematopoietic stem cell homing. J Investig Dermatol Symp Proc 9(3):215–223. https://doi.org/10.1111/j.0022-202X.2004.09301.x

Schneider MR, Schmidt-Ullrich R, Paus R (2009) The hair follicle as a dynamic miniorgan. Curr Biol 19(3):R132–R142. https://doi.org/10.1016/j.cub.2008.12.005

Shpichka A, Butnaru D, Bezrukov EA, Sukhanov RB, Atala A, Burdukovskii V, Zhang Y, Timashev P (2019) Skin tissue regeneration for burn injury. Stem Cell Res Ther 10(1):94. https://doi.org/10.1186/s13287-019-1203-3

Stenn KS, Paus R (2001) Controls of hair follicle cycling. Physiol Rev 81(1):449–494. https://doi.org/10.1152/physrev.2001.81.1.449

Suh W, Kim KL, Kim JM, Shin IS, Lee YS, Lee JY, Jang HS, Lee JS, Byun J, Choi JH, Jeon ES, Kim DK (2005) Transplantation of endothelial progenitor cells accelerates dermal wound healing with increased recruitment of monocytes/macrophages and neovascularization. Stem Cells 23(10):1571–1578. https://doi.org/10.1634/stemcells.2004-0340

Sullivan KM, Goldmuntz EA, Keyes-Elstein L, McSweeney PA, Pinckney A, Welch B, Mayes MD, Nash RA, Crofford LJ, Eggleston B, Castina S, Griffith LM, Goldstein JS, Wallace D, Craciunescu O, Khanna D, Folz RJ, Goldin J, St Clair EW, Seibold JR, Phillips K, Mineishi S, Simms RW, Ballen K, Wener MH, Georges GE, Heimfeld S, Hosing C, Forman S, Kafaja S, Silver RM, Griffing L, Storek J, LeClercq S, Brasington R, Csuka ME, Bredeson C, Keever-Taylor C, Domsic RT, Kahaleh MB, Medsger T, Furst DE, Investigators SS (2018) Myeloablative autologous stem-cell transplantation for severe scleroderma. N Engl J Med 378(1):35–47. https://doi.org/10.1056/nejmoa1703327

Takahashi K, Yamanaka S (2013) Induced pluripotent stem cells in medicine and biology. Development 140(12):2457–2461. https://doi.org/10.1242/dev.092551

Taub AF, Pham K (2018) Stem cells in dermatology and anti-aging care of the skin. Facial Plast Surg Clin North Am 26(4):425–437. https://doi.org/10.1016/j.fsc.2018.06.004

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM (2000) Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102(4):451–461. https://doi.org/10.1016/s0092-8674(00)00050-7

Thakur V, Kumar S, Kumaran MS, Kaushik H, Srivastava N, Parsad D (2019) Efficacy of transplantation of combination of noncultured dermal and epidermal cell suspension vs epidermal cell suspension alone in vitiligo: a randomized clinical trial. JAMA Dermatol 155(2):204–210. https://doi.org/10.1001/jamadermatol.2018.4919

Toma JG, McKenzie IA, Bagli D, Miller FD (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23(6):727–737. https://doi.org/10.1634/stemcells.2004-0134

Uddin F, Rudin CM, Sen T (2020) CRISPR gene therapy: applications, limitations, and implications for the future. Front Oncol 10:1387. https://doi.org/10.3389/fonc.2020.01387

Vaculik C, Schuster C, Bauer W, Iram N, Pfisterer K, Kramer G, Reinisch A, Strunk D, Elbe-Burger A (2012) Human dermis harbors distinct mesenchymal stromal cell subsets. J Invest Dermatol 132(3 Pt 1):563–574. https://doi.org/10.1038/jid.2011.355

Venugopal SS, Yan W, Frew JW, Cohn HI, Rhodes LM, Tran K, Melbourne W, Nelson JA, Sturm M, Fogarty J, Marinkovich MP, Igawa S, Ishida-Yamamoto A, Murrell DF (2013) A phase II randomized vehicle-controlled trial of intradermal allogeneic fibroblasts for recessive dystrophic epidermolysis bullosa. J Am Acad Dermatol 69(6):898–908 e897. https://doi.org/10.1016/j.jaad.2013.08.014

Verfaillie CM, Pera MF, Lansdorp PM (2002) Stem cells: hype and reality. Hematology Am Soc Hematol Educ Program 369–391. https://doi.org/10.1182/asheducation-2002.1.369

Wagner JE, Ishida-Yamamoto A, McGrath JA, Hordinsky M, Keene DR, Woodley DT, Chen M, Riddle MJ, Osborn MJ, Lund T, Dolan M, Blazar BR, Tolar J (2010) Bone marrow transplantation for recessive dystrophic epidermolysis bullosa. N Engl J Med 363(7):629–639. https://doi.org/10.1056/NEJMoa0910501

Wettstein R, Savic M, Pierer G, Scheufler O, Haug M, Halter J, Gratwohl A, Baumberger M, Schaefer DJ, Kalbermatten DF (2014) Progenitor cell therapy for sacral pressure sore: a pilot study with a novel human chronic wound model. Stem Cell Res Ther 5(1):18. https://doi.org/10.1186/scrt407

Wong T, Gammon L, Liu L, Mellerio JE, Dopping-Hepenstal PJ, Pacy J, Elia G, Jeffery R, Leigh IM, Navsaria H, McGrath JA (2008) Potential of fibroblast cell therapy for recessive dystrophic epidermolysis bullosa. J Invest Dermatol 128(9):2179–2189. https://doi.org/10.1038/jid.2008.78

Yang R, Wang J, Chen X, Shi Y, Xie J (2020) Epidermal stem cells in wound healing and regeneration. Stem Cells Int 2020:9148310. https://doi.org/10.1155/2020/9148310

Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z (2019) Stem cells: past, present, and future. Stem Cell Res Ther 10(1):68. https://doi.org/10.1186/s13287-019-1165-5

Zhang J, Chopp M (2013) Cell-based therapy for ischemic stroke. Expert Opin Biol Ther 13(9):1229–1240. https://doi.org/10.1517/14712598.2013.804507

Zhang CP, Fu XB (2008) Therapeutic potential of stem cells in skin repair and regeneration. Chin J Traumatol 11(4):209–221. https://doi.org/10.1016/s1008-1275(08)60045-0

Zokaei S, Farhud DD, Keykhaei M, Zarif Yeganeh M, Rahimi H, Moravvej H (2019) Cultured epidermal melanocyte transplantation in vitiligo: a review article. Iran J Public Health 48(3):388–399. https://www.ncbi.nlm.nih.gov/pubmed/31223565

Current Advances and Future Avenues in Endocrinology

3

Liza Das and Sanjay Kumar Bhadada

Abstract

Endocrinology is a dynamic science with numerous advances in the field of diagnosis, prognosis and management. Newer diagnostic modalities in the field have not only revolutionised the manner glycaemic status in diabetes is assessed but have provided newer metrics of evaluation, including 'time in range' and the importance of glycaemic variability as an independent association with vascular complications. The focus on lifestyle management for weight and glycaemic optimisation is at an all-time high, especially in terms of time-restricted feeding, intermittent fasting and chrononutrition. Precision and personalised medicine is also foraying into mainstream endocrinology, with potential applications in diabetes mellitus as well as other disorders such as acromegaly and adrenal diseases (phaeochromocytoma/paraganglioma). Genetic testing for clinical and predictive endocrinology is another rapidly advancing domain with use in disease gene identification and discerning the genetic and molecular basis of various endocrine disorders. Avenues for the future implicate improved genetics, epigenetics and environmental factors to understand the intricacies of disease as well as design more effective therapeutic options.

Keywords

Chrononutrition · Precision medicine · Metabolic surgery · Predictive genetics · COVID-19

L. Das · S. K. Bhadada (✉)
Department of Endocrinology, PGIMER, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_3

3.1 Introduction

Endocrinology is the science and art of hormones. The current era has seen rapid strides in the progress of the science especially in the fields of diabetes monitoring and insulin delivery, changing the paradigm from care to cure of diabetes, the concepts of precision medicine to design pathogenesis-driven tailor-made treatment options for these patients. The epidemic of diabetes is driven by the twin epidemic of obesity and in this context, the concepts of 'chrononutrition' including time-restricted feeding, intermittent fasting as well as pharmacotherapy including pleiotropic agents (GLP1 agonists, SGLT2 inhibitors) and surgical management (bariatric surgery) have gradually positioned themselves in the treatment algorithms for patients battling both these conditions. The other area with an ever-increasing burden of disease, primarily associated with increased longevity, is osteoporosis. Effective treatment options for this condition are increasingly being recognised and used in clinical settings to improve bone health and prevent morbidity and mortality associated with fragility fractures. The year has been a particularly momentous time for healthcare globally with the huge impact of COVID-19, and endocrinology is no exception. It is now reasonably well established that multiple chronic conditions with an endocrinological basis, including diabetes mellitus, obesity and hypertension, portend poor prognosis in patients with COVID-19. It is also being increasingly identified that new-onset diabetes and involvement of other endocrine organs, including the pituitary, thyroid, pancreas, adrenals and gonads, by COVID-19 are common and their timely diagnosis and treatment may possibly have a bearing on patient outcomes.

3.2 Diabetes Mellitus

Diabetes mellitus is a pandemic in its own right and the ever-increasing burden of disease has undoubtedly led to significant adverse implications on health, society and economics. However, it is this onus that has and will continue to provide impetus for scientific, translational and clinical research in terms of better disease diagnosis and management.

3.2.1 Islet Cell Biology/Transdifferentiation

Preservation and/or restoration of functional β-cell mass is an important therapeutic goal in the management of diabetes mellitus. However, the limited regeneration potential of β-cells in the islets is a major impediment in this direction thereby directing the focus to reprogramme other available functional cells to functional β-cells. Active scientific pursuit has shown that α-cells, owing to their developmental and positional similarity, can be engineered to transform into insulin producing β-cells by exploiting their 'plasticity potential', without any significant adverse effects due to loss of α-cells.

3.2.2 Biomarkers in Diabetes

In a recent review of all biomarkers for the diagnosis of prediabetes and diabetes, various microRNAs were found to be useful in predicting diabetes onset and progression. MicroRNAs are small, non-coding RNAs that act by gene silencing/suppressing translation. miR-375 was one of the earliest markers associated with β-cell injury and chronic hyperglycaemia, with levels being elevated till disease onset and decreasing later on. This suggested its potential utility as a biomarker to predict onset of diabetes in high-risk individuals. miR-23a and miR-126 were later found to be lower in patients with DM or prediabetes (Lees et al. 2017).

Apart from microRNAs which have a more translational impact, the role of metabolomics is important from the translational and clinical point of view in diabetes. Metabolomics is the branch of omics that deals with the ultimate metabolic constitution of the cell and represents the most integrated profile as it is the net result of genomics and transcriptomics. Branched chain amino acid (BCAA) and aromatic amino acids like phenylalanine, tyrosine, valine, leucine and isoleucine have been detected to be directly and glycine and glutamate inversely associated with T2DM. BCAAs activate mTORC1 and the downstream pattern S6 kinase1, which ultimately interferes with IRSs by causing its serine phosphorylation. These changes drive insulin resistance despite similar weight gain in different individuals, as elegantly demonstrated in rodent models. Among lipids, lower carbon number and lower double bond content were found to confer a higher risk even after 7 years of follow-up. Increased levels of lactate, glycolytic intermediates and decreased levels of TCA intermediates are also part of the metabolomic signature of T2DM. Another important intermediary metabolite is 2-AAA, or aminoadipic acid, which has been found to predict diabetes over a long follow-up of 12 years in normoglycaemic individuals. The specific association of this intermediate was with fasting insulin, suggesting its bearing on insulin resistance. Apelin was the other metabolite identified as being associated with diabetes. Apelin is an adipokine and a natural ligand of the G-protein-coupled receptor APJ, expressed in various tissues.

3.2.3 Advances in Glucose Monitoring

The method of continuous glucose monitoring is based on the concept of measuring glucose in interstitial fluid and has the advantage of having data for short-term use as well for monitoring trends over the longer term. Patients place their device every 10–14 days, depending on the brand/manufacturer of the device they are using. This improves compliance and minimises missing data, inherent to self-monitoring of blood glucose. The Medtronic and Dexcom continuous glucose monitoring systems not only allow real-time monitoring but also provide alerts for hypoglycaemia to enable timely action for averting serious complications as well for hyperglycaemia above the preset target ranges. The flash glucose monitoring system or Freestyle Libre enables real-time assessment of multiple glucose values throughout the day without additional pricks. This generates multiple values, over and above the

conventional SMBG, which can enable identification of previously undetected (and asymptomatic) hypoglycaemic episodes or undue patterns of hyperglycaemia. Multiple postprandial values can aid in treatment decisions, to make more informed decisions on food choices and portions. The other clinical utility of these measurements is monitoring of glucose during the night, especially in patients with brittle glycaemic control or repeated night-time hypoglycaemia. The bottom line of these devices is that they encourage and enable more frequent as well as convenient monitoring of glucose, detect unrecognised glycaemic perturbations and enable assessment of 'time-in-range', so as to improve overall glycaemic control and delay complications.

3.2.4 Advances in Insulin Delivery

Insulin pumps represent an important technology designed for people with uncontrolled glucose profile despite multiple subcutaneous insulin regimens or those with brittle glucose control (Nimri et al. 2020). Their specific advantages include the following:

(a) Improved adherence to insulin therapy
(b) A delivery method that is more physiological and that allows for delivery of more precise doses
(c) The achievement of reduced variability in glucose levels throughout the day due to the ability to adjust settings based on the personalised needs of the individual patient
(d) Flexible management of diabetes
(e) Tailoring insulin to meet daily insulin requirements and circadian variation
(f) Data analysis for treatment optimisation
(g) Alternative for patients with T1DM who do not reach therapy goals despite adherence

There is evidence to suggest definite improvement in quality of life and reduced insulin requirements with the use of pump therapy (Fig. 3.1). (Sora et al. 2019). Information on reduction of glycaemic burden and perturbations in glucose patterns (hypoglycaemia and ketoacidosis) as compared to multiple subcutaneous insulin injections is less consistent (Table 3.1).

3.2.5 Chronomedicine

Chronomedicine refers to that aspect of medicine which deals with the importance of circadian rhythms. Central (suprachiasmatic nucleus of hypothalamus) and peripheral molecular clocks respond to external cues including temperature, feeding/fasting cycles, exercise and hormone function to optimise numerous physiological and behavioural responses over a 24-h cycle. Disturbances in these rhythms are associated with metabolic dysfunction, obesity, type 2 diabetes and other metabolic states.

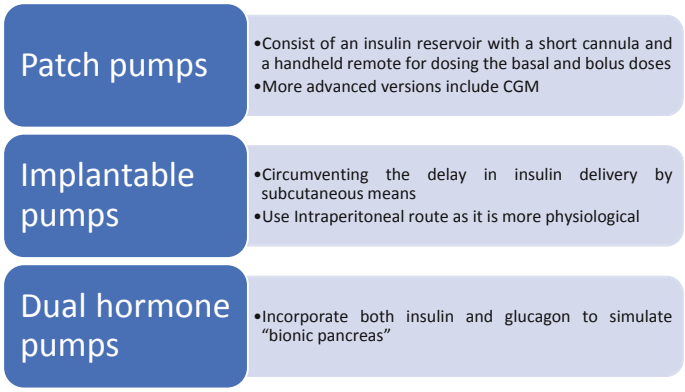


Fig. 3.1 Schematic representation of types of insulin pumps

Table 3.1 Summary of evidence comparing insulin pump with multiple subcutaneous insulin therapy

Parameter	Insulin pump	MDI/MSII
HbA1c	Better	Similar to better
Severe hypoglycaemia	Similar	Inconclusive
DKA	May be higher (in cases of pump failure)	Similar
Quality of life	Improved	Improved
Insulin requirement	Lower requirement	Lower requirement

DKA diabetic ketoacidosis, *HbA1c* glycated haemoglobin, *MDI* multiple daily insulin, *MSII* multiple subcutaneous insulin infusion
Adapted from Misso et al. Cochrane database systems review. 2010 and Nimri et al. American journal of therapeutics. 2019

Melatonin is a hormone closely linked with the sleep-wake cycle. SNPs associated with melatonin receptors have been found to be associated with diabetes, but the strength of the association needs to be studied further. Therapies targeting these receptors, especially MT2R, are being channelised for clinical studies in preventing diabetes. Other hormones, especially insulin and IGF1, also work by inducing the repressors of circadian rhythm, thereby providing a mechanism by which feeding cues synchronise biological clocks throughout the body due to the widespread distribution of these hormones and their receptors across the body. The clinical implications of these findings lie in the fact that this may be a key physiological driver underlying the association between disturbances in circadian rhythms (shift-work, jet lag) and ill health.

The other clinical application of this concept extends to 'chrononutrition', in which therapeutic use of 'time-restricted feeding' or limiting the food cycle to 8–12 h and observing fasting in the remainder of the time have shown remarkable benefits in terms of the amelioration of obesity-associated metabolic disorders in rodent models as compared to mice fed an isocaloric diet ad libitum. The reasoning behind this benefit was attributed to the reduction in cellular stress and enhanced

synchrony between peripheral cues and neurohormonal mechanisms governing the sleep-wake cycle.

3.2.6 Precision Medicine in Diabetes

This is a relatively new concept in the integrated management of various diseases, including diabetes. It includes various factors that may impact the ultimate disease onset, progression and phenotype of diabetes in an individual patient. Though inexorable loss of functional β-cell mass is the culmination of various pathophysiological pathways in the etiopathogenesis of diabetes, there is considerable variation in the factors that predispose to it, including genetic and non-genetic factors like epigenetics, environment, lifestyle and multiple omics, inherent to an individual.

Therefore, current understanding of diabetes management is that there is no single therapy or line of management that is optimal for all patients (Mohan and Radha 2019). Rather, any given patient may be identified by the major pathophysiological mechanisms operating in him/her and therapy tailor made for his/her predominant metabolic dysregulation pathway (Fig. 3.2).

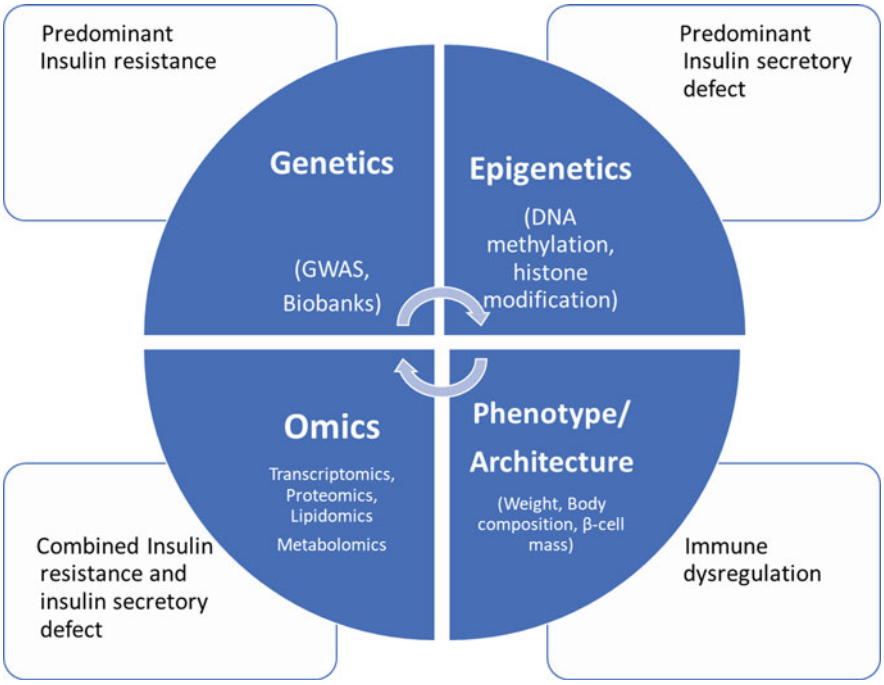


Fig. 3.2 Interplay of various pathogenic mechanisms and processes in an individual with diabetes leading to predominant disease phenotype in him/her

3.2.7 The Journey from Diabetes Care to Diabetes Cure

β-cell dysfunction or failure is no longer an irreversible phenomenon. Multiple lines of evidence which support this fact include bariatric surgery or 'metabolic surgery', lifestyle measures like calorie restriction to induce 10–15% loss of basal weight, intermittent fasting including time-restricted feeding and inducible pluripotent stem cells for transdifferentiation (Taylor et al. 2019). The most cost-effective and least invasive method of all these options is the negative energy balance which was shown to reduce ambient glycaemia and insulin resistance as early as within 1 week of intervention and found to be sustained till 6 months duration. The demonstration of restoration of the first-phase insulin response was a big pointer towards the phenomenon of reversal with diet alone.

Bariatric surgery is another modality touted as being a curative option for diabetes. Though evidence is reasonable even for long-term diabetes remission, the greatest beneficiaries are the younger population, those with a shorter duration of diabetes, better preoperative glycaemic control and not on insulin prior to surgery. There is still a long way to go before surgery can be declared as the cure for diabetes, and it certainly is a viable option for overweight/obese individuals who have failed a trial of lifestyle and other pharmacotherapeutic measures and may help in providing synergistic benefits with existing medical options. The safe option currently is to use metabolic surgery as a complementary rather than competitive approach to medical management.

3.2.8 Non-bariatric Metabolic Surgery for Diabetes Mellitus

The basis of this concept is derived from the fact that remission of diabetes following bariatric surgery is partially independent of weight loss as it can happen earlier and irrespective of weight loss. This suggested the role of hormonal mechanisms including incretins that may have a role in diabetes remission. Omentectomy for visceral adiposity and electrode stimulation have shown partial success in diabetes remission. Endoscopic ablation of the duodenal mucosa followed by mucosal regeneration is another promising therapy with possible mechanisms involving reduction in hepatic glucose output and improving insulin resistance (Zierath 2019).

3.3 Osteoporosis

Osteoporosis has transitioned from an inevitable consequence of ageing to a potentially preventable and treatable lifestyle disease. As a result, there are various categories of drugs available for the effective management of disease in the long term (Cairoli et al. 2015). The goals of treatment include improvement in bone mass and prevention of fractures. Anti-osteoporotic therapies may be either anti-resorptive, osteoanabolic or have mixed effects (Khosla and Hofbauer 2017). Their development strategies have varied from incidental discovery to dedicated scientific research, summarised in Table 3.2.

Table 3.2 Various groups of drugs used for osteoporosis and the pathway leading to their development

Category of drug	Drug name	Drug development journey
Anti-resorptive		
Bisphosphonates	Zoledronate, pamidronate, alendronate	Serendipitous discovery of positive effects on bone from industrial compounds
Anti RANKL	Denosumab	Scientific/translational research
Calcitonin	Calcitonin	Physiological research
Oestrogen	Oestrogen	Clinical observations
SERM	Raloxifene, bazedoxifene	Medical research
Osteoanabolic		
PTH	Teriparatide	Animal studies followed by human trials
PTHrP analogues	Abaloparatide	Animal studies followed by human trials
Mixed anti-resorptive and anabolic		
Sclerostin antibody	Romosozumab	Derived from studies on rare bone diseases
Cathepsin K inhibitor	Odanacatib	Derived from studies on rare bone diseases

PTH parathyroid hormone, *PTHrP* parathyroid hormone-related peptide, *RANKL* receptor activator of nuclear factor κ B, *SERM* selective oestrogen receptor modulator

Other therapeutic targets which can be exploited as potential molecules for improving bone health include the following:

3.3.1 Molecules from Basic Research

- **Nitric oxide (NO):** This molecule acts by inducing cyclic GMP pathway which activates protein kinase G. Soluble guanylate cyclase inhibitors have been proven to be efficacious in ovariectomised animal models to improve bone health and microarchitecture, thereby suggesting its potential role in postmenopausal osteoporosis.
- **Sirtuin:** Sirt1 is an NAD-dependent acetylase involved in multiple physiological processes. Rodent models with Sirt1 deficiency resemble a low bone mass phenotype due to poor bone formation. Resveratrol, a phytoestrogen, is a Sirt1 activator and has been found to be of use in clinical studies.
- **Tryptophan hydroxylase inhibitor:** Tryptophan hydroxylase is the enzyme catalysing the synthesis of serotonin. Serotonin in preclinical and clinical models has been found to increase bone mass and bone formation markers as well as improve bone architecture.
- **miRNAs:** MicroRNAs have a putative role in osteoblastogenesis and bone formation. Development of new miRNA mimics or inhibitors to target skeletal health is an exciting area of ongoing and future research.

- **Activin A:** Activin, a member of the TGF-β family, has a role in promoting osteoclastogenesis and inhibiting osteoblast differentiation. Antagonists of activin A have a putative role in inhibiting osteoclast activation and therapy in osteoporosis.
- **11βHSD1 inhibitors:** 11βHSD1 is the enzyme that catalyses the formation of cortisol from cortisone. Therefore, inhibitors of 11βHSD1, which reduce the circulating and local levels of endogenous steroids, have potential in being used for osteoporosis, especially diabetes-associated osteoporosis.
- **Semaphorins:** These are small molecules, especially Sema 3A, which is secreted by both osteoclasts and osteoblasts and have a dual role in not only osteoclast inhibition but also osteoblastogenesis. Rodent models have demonstrated their efficacy in reducing loss of bone mass.

3.3.2 Molecules with Proposed Use as Repurposed Therapies

- **GLP1:** This is an incretin secreted by the L cells of the small intestine, which act on the islets of pancreas to secrete insulin. There is another isoform of the receptor on osteoblasts, which is involved in improvement in bone mass. In line with this concept, there is demonstrable evidence to suggest the improvement of bone mass with the use of GLP1 agonists, including liraglutide.
- **Vitamin K:** Vitamin K is involved in the γ-carboxylation of osteocalcin, a bone formation marker. There is recent preliminary evidence to suggest the usefulness of vitamin K supplementation in improving BMD and fracture prevention.

3.4 Obesity

Obesity is a relapsing chronic disease that has attained the proportions of a pandemic and in combination with diabetes and has made the entity of diabesity an unsurmountable challenge. The principles of management of obesity are lifestyle measures followed by pharmacotherapy and surgery. However, lifestyle measures provide the most durable and cost-effective intervention to target this growing pandemic. The tenets of lifestyle measures include diet and physical activity, both of which have individual and synergistic roles for attaining and maintaining weight loss in overweight and obese individuals.

3.4.1 Dietary Patterns

Advancements in these lifestyle measures for the prevention and treatment of overweight/obesity include continuous energy restriction (CER) or intermittent fasting (IF) as a therapeutic modality in these patients. CER and IF are both time-

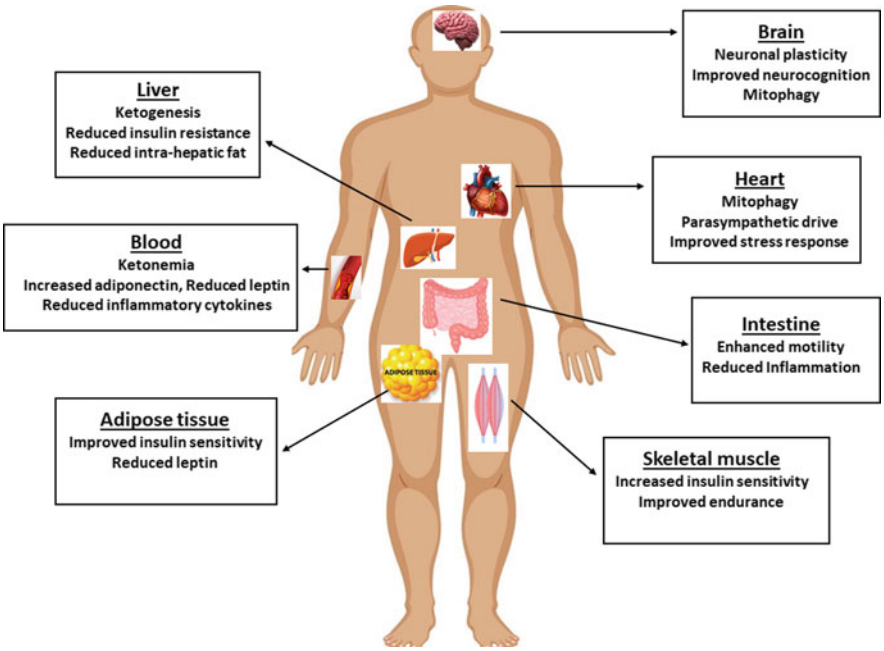


Fig. 3.3 Benefits of time-restricted feeding or intermittent fasting used as a therapeutic modality in the management of diabetes and obesity

tested methods, but the real mechanisms underlying their efficacy are being unravelled not only in terms of weight overall but also body composition (lean mass), ectopic fat deposition (liver, pancreas, pericardium) and glycaemic homeostasis (Brown et al. 2013).

IF has been found to be similar to CR in terms of weight loss, weight maintenance and improved cardiovascular risk factors at 1 year following intervention with better preservation of lean mass than CR diet. Plausible underlying mechanisms involved in metabolic benefits with IF include redistribution of fat, weight loss, relatively preserved lean mass, browning or beigeing of white adipose tissue and favourable alteration of gut microbiota (Fig. 3.3).

3.4.2 Pharmacotherapy

Among pharmacotherapies for obesity, newer options available include oral GLP1 agonists and SGLT2 inhibitors. Though clinical experience with GLP1 agonists and SGLT2 inhibitors is almost a decade old, newer mechanisms are being unearthed, which explain their superior efficacy in overall metabolic health, including weight, glucose and blood pressure homeostasis as well as unprecedented cardiovascular and

renal benefits. Oral GLP1 agonists including semaglutide have been found to be non-inferior in terms of weight loss and cardiovascular efficacy. SGLT2 inhibitors have also revolutionised the way we treat diabetes, especially in overweight/obese individuals. They have now gained second position in the treatment algorithm of diabetes mellitus and to be used as first choice in patients with heart failure. Their efficacy even in individuals without diabetes has helped to pave the way for their use as treatment options for heart failure, NASH, PCOS and other metabolic diseases.

3.4.3 Translational Research

MC4R or melanocortin 4 receptor is a G-protein-couple receptor that is mostly involved in the weight regulation, and its mutations resulting in its deficiency are the most common cause of early onset severe obesity. Recent research identified nearly 60 variants in this gene in the UK Biobank (half a million population) and found that MC4R gain-of-function was associated a lean phenotype or 'thinness', lower BMI as well as lower risk of diabetes mellitus, obesity and coronary artery disease. This basic research enabled the development of setmelanotide, an oral MC4R agonist, for the clinical use. Though data is now available mostly for genetic obesity, it is likely that the potential of these agents will be tapped in the future for overweight/obesity of a nonsyndromic variety.

3.5 Genetics in Endocrinology

Endocrinology is witnessing a sea change in the field of genetic testing for use in clinical and predictive settings. With the application of newer and advanced techniques including NGS (next-generation sequencing), there is an ever increasing portfolio of tests being used for monogenic disorders that result in single or multiple endocrine gland involvement for disease gene identification and delving deeper into the genetic and molecular basis of various endocrine disorders (Table 3.3). The utility of these techniques not only has diagnostic implications for a given individual ('diagnostic genetics') but also the family, especially first- and second-generation relatives, who can be identified early using cascade screening protocols, ('predictive genetics'.) (Newey 2019). Endocrine disease can be monogenic or polygenic, but the genetic architecture of an individual has a more significant bearing in case of monogenic disorders.

3.5.1 Genetic Aberrations

Table 3.3 Utility and clinical applications of the types of genetic testing as applied to endocrine disease

Parameter	First-generation sequencing	Next-generation sequencing
Type of testing	Single-/pauci-gene panel	Based on multiple parallel testing Multiple gene panel Disease-targeted/WES/WGS
Number of genes tested	Up to 5 usually	>5 (up to 20,000 in WGS)
Number of nucleotides	Up to 10,000 usually	>10,000 (up to 300 million)
Detection of variants	Usually low detection as it is specific	High detection of variants, including VUS which require bioinformatics analysis
Clinical indications	Monogenic disorders	Monogenic disorders with high genetic heterogeneity or unknown genetic background
Target regions sequenced	Exons and intron/exon boundaries of genes being tested	Exons and intron/exon boundaries of genes being tested or whole exome (all exons and intron/exon boundaries) or whole genome (all exons and introns)
Type of genetic abnormality detected	SNPs Indels	SNPs Indels CNV Translocations/rearrangements in WGS

WES whole exome sequencing, *WGS* whole genome sequencing, *VUS* variant of unknown significance, *SNP* single nucleotide polymorphisms, *CNV* copy number variants, *Indel* insertions and deletions

(Adapted from Newey et al. Clinical Endocrinology. 2019)

3.5.2 Chromosomal Aberrations

Karyotyping is the conventionally available genetic tool for diagnosing disorders associated with chromosome number (aneuploidy) or structure (copy number variation, inversions or translocations). Array CGH represents the advancement of this technique and is of use in detecting large chromosomal deletions or translocations. Additional advanced methods to detect structural changes include the following:

- FISH—to detect specific chromosomal translocations
- Digital droplet PCR—to detect CNV
- MLPA—useful for partial or whole gene deletions (MEN1, VHL)

3.5.3 Current Applications and Future Uses

- **Preimplantation/prenatal genetic counselling**: Preimplantation counselling in case of IVF or prenatal testing in case of normal pregnancy to detect transmission of hereditary disease. The advancement in this arena is the use of non-invasive testing, including cell free circulating foetal DNA in the maternal circulation for detecting aneuploidy or X-linked disorders.
- **Somatic mutation testing for endocrine oncology**: For identification of somatic or non-germline mutations in various endocrine cancers. The implications of this testing involves not only diagnostic but also prognostic and therapeutic benefits. Identification of tumour-specific mutations such as BRAF V600E for craniopharyngiomas or RET mutations for MTC can provide opportunity for therapy using tyrosine kinase inhibitors. Profiling the genetic architecture of several different conditions have led to the identification of somatic mutations in pituitary adenomas (corticotropinomas, USP8) or aldosterone-producing adenomas (KCNJ5).
- **Liquid biopsy:** The use of cell-free circulating DNA is gaining importance in endocrine genetics not only in terms of prenatal testing but also as a minimally invasive method for diagnosing various genetic mutations in terms of neuroendocrine and other tumours. Other potential applications include disease stratification, prognostication and surveillance.
- **Future Challenges**
  There is a discordance between the rate of advancement in sequencing technologies and the clinical applications of the huge quantum of information, especially the huge burden of evidence on genetic variation in the normal population. The challenges in the future will be to delve deeper into the realms of unravelling the nuances of the impact of genetic architecture on disease phenotypes in a given individual.

  The interaction of genetics with the environment or epigenetic modifiers also remains to be understood more and more in the context of endocrine diseases to understand the complexities of disease phenotype and expressivity.

  Application of these techniques to population-based screening will enable identification of asymptomatic carriers or presymptomatic individuals but will undoubtedly be a social and economic burden on the endocrine community. This challenge needs to be met by increased allocation of dedicated resources and resource persons for the same.

3.6 COVID-19 and Endocrinology

Covid-19, caused by the SARS-CoV-2, is a pandemic and an immense challenge to global healthcare. The sustained human-to-human transmission has led to overwhelming spread of the disease, and despite a low case fatality rate as compared to other known coronavirus epidemics (SARS, MERS), the absolute numbers of infected population has led to global mortality exceeding two and a half lakh individuals. Despite similar rates of susceptibility to the infection, it is now well

established that the host immune response plays a major role in disease outcome. Diabetes, hypertension and obesity have emerged as the major modifiers of response to SARS-CoV-2 infection. These chronic conditions have a definitive endocrine association, thereby suggesting the potential impact of endocrine dysfunction on Covid-19 outcomes.

Multiple endocrine axes including hypothalamic-pituitary-adrenal, gonadal and thyroid are affected in patients with Covid-19. These findings have been substantiated further by the observations of more severe endocrine dysfunction in patients with more severe COVID-19 (Das et al. 2021). Though logical due to several inflammatory endocrine syndromes caused due to viral aetiologies, overall clinical evidence of endocrine involvement of multiple axes in patients with Covid-19 is scarce. Whether they are due to direct cytotoxicity of the virus, cytokine storm or related to ACE2 expression (entry receptor for the SARS-CoV-2) remains to be investigated.

Evidence at present pertaining to endocrine dysfunction is mostly speculative and extrapolated from evidence available in patients with SARS-CoV. Moreover, a lot of this evidence is from autopsy data and is hence, retrospective. Well-designed prospective studies specifically addressing the knowledge gap in patients with Covid-19 are needed but are lacking at present. The clinical implications of these findings can provide evidence-based guidelines for various facets of patient management including glucocorticoid supplementation and evaluation for gonadal function, especially in reproductive age group males and thyroid and pituitary functions at baseline as well as during recovery so as to avert potentially fatal complications arising due to unrecognised hormone deficiencies.

Diabetes predisposes to severe disease in Covid-19 but there is also postulation that the infection can induce hyperglycaemia by multiple mechanisms. The 'cytokine storm' described as the hallmark of severe Covid-19, mediated by pro-inflammatory cytokines including IL-6, TNF-α and IL-1, can acutely impair insulin sensitivity and cause hyperglycaemia. Pro-inflammatory cytokines are known to reduce the mRNA of insulin receptor substrate-1 (IRS-1), thereby interfering with post-receptor hormone signalling. They are also known to promote lipolysis in peripheral adipose tissue, thereby leading to the generation of excess free fatty acids, which can interfere with insulin action of glucose uptake into cells (lipotoxicity). ACE2 is the functional receptor for the SARS coronavirus that facilitates entry of the virus into the alveolar epithelial cells. However, ACE2 abundance is also demonstrated on pancreatic islets and exocrine pancreas. The SARS coronavirus directly binds to ACE2 receptors on pancreatic islets causing acute hyperglycaemia by impairing insulin secretion from the islets. Pancreatitis (by viral entry mediated damage), based on elevations in lipase and amylase, has been reported in over one-sixth of a cohort ($n = 52$) hospitalised with SARS-CoV-2-associated pneumonia. There is also the suggestion of an underlying genetic predisposition for infection by SARS-CoV-2 due to ACE2 polymorphisms which have been linked to diabetes and hypertension in certain ethnic groups. Therefore, there is preclinical and clinical evidence pointing towards both impaired insulin secretion and reduced insulin sensitivity in patients with Covid-19 (Fig. 3.4).

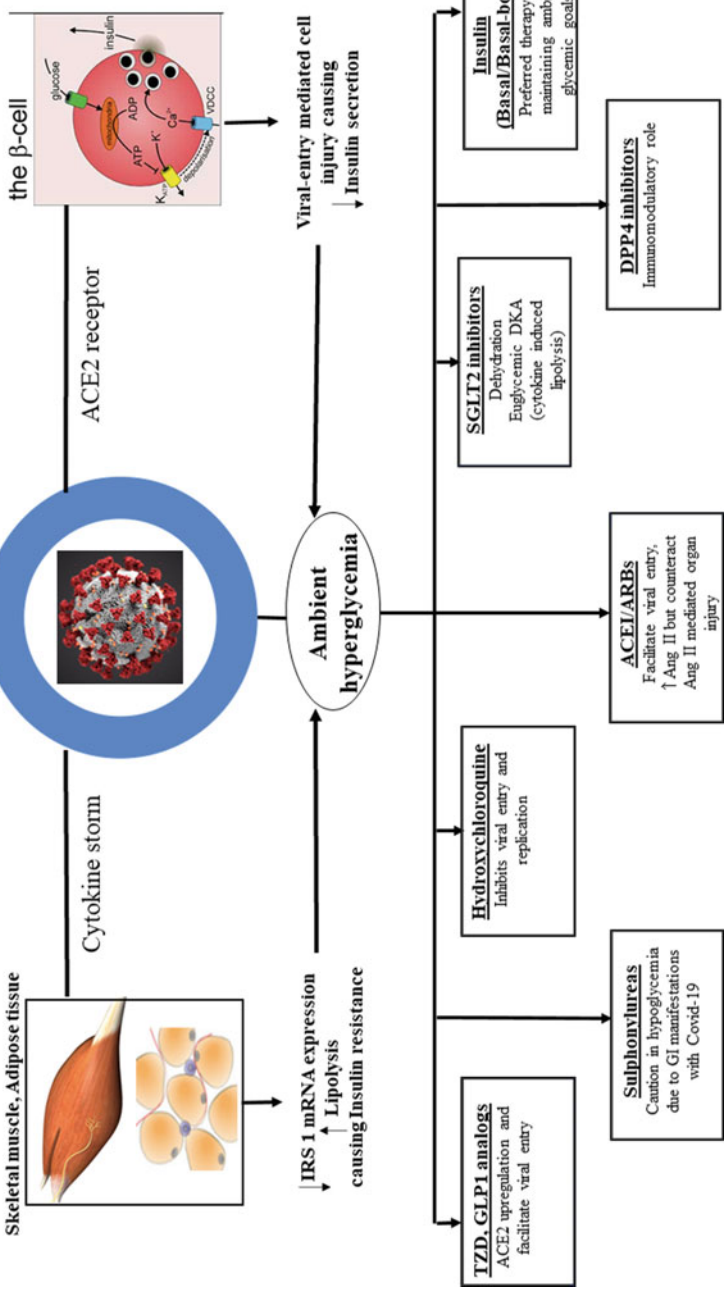


Fig. 3.4 Schematic representation of possible mechanisms of diabetes onset or aggravation in patients with COVID-19 and proposed use of various anti-diabetic agents for management

3.7 Conclusion

Current advances in endocrinology have enabled better modalities of monitoring and drug delivery in diabetes, identification of invasive (bariatric surgery) and non-invasive (calorie restriction, time-restricted feeding) methods for diabetes treatment and the use of technology for overall management of diabetes, obesity and osteoporosis. There has been no greater clinical relevance of personalised medicine and genetics in endocrinology than is being used now. The future holds multiple prospects for improvement in integration of genetics, epigenetics and environmental factors to understand the intricacies of disease as well as design more effective therapeutic options.

References

Brown JE, Mosley M, Aldred S (2013) Intermittent fasting: a dietary intervention for prevention of diabetes and cardiovascular disease? Br J Diabetes Vasc Dis 13(2):68–72

Cairoli E, Zhukouskaya VV, Eller-Vainicher C, Chiodini I (2015) Perspectives on osteoporosis therapies. J Endocrinol Investig 38(3):303–311

Das L, Dutta P, Bhadada SK, Bhansali A, et al (2021) Spectrum of endocrine dysfunction and association with disease severity in patients with COVID-19: Insights from a cross-sectional observational study. Front Endocrinol (Lausanne) 12:645787

Khosla S, Hofbauer LC (2017) Osteoporosis treatment: recent developments and ongoing challenges. Lancet Diabetes Endocrinol 5(11):898–907

Lees T, Nassif N, Simpson A, Shad-Kaneez F, Martiniello-Wilks R, Lin Y, Jones A, Qu X, Lal S (2017) Recent advances in molecular biomarkers for diabetes mellitus: a systematic review. Biomarkers 22(7):604–613

Mohan V, Radha V (2019) Precision diabetes is slowly becoming a reality. Med Princ Pract 28(1): 1–9

Newey PJ (2019) Clinical genetic testing in endocrinology: current concepts and contemporary challenges. Clin Endocrinol 91(5):587–607

Nimri R, Nir J, Phillip M (2020) Insulin pump therapy. Am J Ther 27(1):e30–e41

Sora ND, Shashpal F, Bond EA, Jenkins AJ (2019) Insulin pumps: review of technological advancement in diabetes management. Am J Med Sci 358(5):326–331

Taylor R, Al-Mrabeh A, Sattar N (2019) Understanding the mechanisms of reversal of type 2 diabetes. Lancet Diabetes Endocrinol 7(9):726–736

Zierath JR (2019) Major advances and discoveries in diabetes-2019 in review. Curr Diab Rep 19(11):118

Autologous Conditioned Serum in Lumbar and Cervical Radiculopathy: A Systemic Review

4

Praveen Sodavarapu, Vijay G. Goni, Akash Ghosh, Sandeep Patel, Vishal Kumar, and Sunil Kumar

Abstract

Background: Intervertebral disc degeneration causing radiculopathy is driven by catabolic cytokines like IL-1β and TNFα. Autologous conditioned serum (ACS) was found to be rich in IL-1Ra (Interleukin-1 Receptor Antagonist), and thus, can impede disc degeneration. A systematic review of available literature was conducted to ascertain the potential therapeutic application of ACS in radiculopathy.

Methods: Systematic literature reviews were conducted in PubMed, Scopus and Embase databases, up to September 2020. Randomised controlled trials (RCTs), prospective, retrospective studies and case series with lumbar or cervical radiculopathy and reporting use of ACS were included, with at least one of the outcome measures like VAS (Visual Analogue Scale) for pain, SF-12 (Short Form of Health Survey-12), Oswestry Disability Index, with a minimum follow up of 3 months. Animal studies, abstracts, review articles and case reports were excluded.

Results: A total of four studies, including 107 patients who received ACS were included based on the eligibility criteria. Two were RCTs and two were prospective non-comparative studies. Three studies evaluated the effect of IL-1Ra on lumbar radiculopathy and one on cervical radiculopathy. The mean age of patients in the studies ranged from 37.15 to 53.9. The dose of ACS used was 2–4 mL injection. In 1 RCT, methylprednisolone was used as control, in the other 5 mg and 10 mg triamcinolone was used. All studies reported a statistically significant reduction in pre-injection and post-injection VAS, there was also a

P. Sodavarapu · V. G. Goni (✉) · A. Ghosh · S. Patel · V. Kumar · S. Kumar
Department of Orthopaedics, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_4

significant difference as compared to 5 mg triamcinolone. Three studies reported significant improvement in ODI. Two studies reported statistically significant improvement in SF-12 scores post injection ($p < 0.001$). For cervical radiculopathy, Neck pain disability score showed a decrease of 73.76% from pre-injection to final follow up and Neck disability index showed a decrease of 74.47%.

Conclusion: All of the four studies concluded that epidural perineural injection with ACS, reduced pain scores (VAS, NPDS) and improved functional scores (ODI, SF-12 and NPDS), as compared to placebo and other conventional therapeutic modalities like steroids, and analgesic-anaesthetic-steroid cocktail. Hence, ACS is a promising new therapeutic modality in both lumbar and cervical radiculopathy, and further studies can strengthen the present evidence regarding its efficacy and safety profile.

Keywords

Chrononutrition · Precision medicine · Metabolic surgery · Predictive genetics · COVID-19

4.1 Introduction

Low back pain is one of the most common presenting complaints that orthopaedic surgeons come across. It affects approximately 23% of the world population, with the majority being females in the age group 40–80 years (Hoy et al. 2012). Neck pain is the fourth leading cause of disability worldwide, with an annual prevalence of more than 30% (Cohen 2015). Lumbosacral radiculopathy and cervical radiculopathy are defined as low back pain and neck pain, respectively, due to irritation or compression of nerve roots, which can arise due to disc herniation, degeneration of intervertebral disc and narrowing of the intervertebral foramen through which the nerve roots exit (Mansfield et al. 2020).

There are multiple treatment strategies for cervical and lumbar radiculopathy, including operative and non-operative. Non-operative measures are mostly applicable in the initial stages where the pathological process can be arrested or reversed. Degeneration of intervertebral disc leads to decrease in intervertebral space, leading therefore to radicular pain. This degenerative process is thought to be due to an inflammatory process, which involves an imbalance between catabolic and anabolic signalling. Catabolic cytokine IL-1β and TNFα are key regulators of intervertebral disc degeneration (Daniels et al. 2017). IL-1 and TNF upregulate catabolic enzymes like A disintegrin and metalloproteinase with thrombospondin motifs −4,5 (ADAMTS) and matrix metalloproteases (MMPs) and decrease expression of anabolic extracellular matrix (ECM) proteins like aggrecan and collagen II, thus they cause breakdown of the ECM and they also cause the cells of the intervertebral disc

to release proinflammatory cytokines which further attract inflammatory cells, thus accelerating the process of degeneration (Johnson et al. 2015). As a result, mitigating the effect of IL-1 and TNF may emerge as a potential treatment strategy for lumbar and cervical radiculopathy.

Anakinra is an IL-1 receptor antagonist (IL-1Ra) derived through recombinant DNA technology from *Escherichia coli*, though it has been used in orthopaedics for over a decade for arthrofibrosis, persistent joint effusion, gout and osteoarthritis, but due to its short lasting action, its use has been limited (Chevalier et al. 2009; Brown et al. 2010). The recent drive in therapeutics has been towards biological and autologous products. One such novel autologous product which has recently been in use is autologous conditioned serum (ACS). Wehling et al. developed the Orthokine® technique of producing ACS, wherein 50–60 mL of venous blood is incubated for 24 h at 37 ° C with medical-grade treated glass beads; it is then centrifuged, and the serum fraction is found to be rich in IL-1Ra. This IL-1 Ra was found to be partly from the breakdown of cells and partly from de novo synthesis by monocytes. This ACS can then be used as an intra-articular injection (Wehling et al. 2007). There have been multiple studies which have shown ACS to be beneficial in equine osteoarthritis model and human knee osteoarthritis. Intra-articular injections of ACS have decreased pain and improved functional scores in patients with osteoarthritis of the knee with a side effect profile comparable to placebo (Wehling et al. 2007; Ajrawat et al. 2019). It reduces the synovial inflammation and arrests cartilage degradation in osteoarthritis (Ajrawat et al. 2019). There are few studies exploring the use of ACS in lumbar and cervical radiculopathy. The effect of ACS in osteoarthritis of the knee could be similar in the therapy of radiculopathy due to age- and inflammation-related disc degeneration. Hence, we review the few studies conducted on this subject to ascertain this potential application of ACS in the future.

4.2 Methods

4.2.1 Literature Search

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (Moher et al. 2009). We searched the electronic databases including PubMed, Scopus and Embase, from the date of inception of database up to 26 September 2020, without any restriction on language. The keywords used for the search were autologous serum, "autologous conditioned serum", interleukin 1, lumbar, cervical, spin* vertebral, radicular, radiculopathy (Table 4.1).

Table 4.1 Search Methodology and the key words used in various databases

Database	Date: 26 Sep 2020	Hits
Pubmed	(((autologous serum OR "autologous conditioned serum" OR interleukin 1)) AND (Lumbar OR cervical OR spin* OR vertebral)) AND (radicular OR radiculopathy) ((("autolog"[All Fields] OR "autologeous"[All Fields] OR "autologic"[All Fields] OR "autological"[All Fields] OR "autologous"[All Fields] OR "autologously"[All Fields]) AND ("serum"[MeSH Terms] OR "serum"[All Fields] OR "serums"[All Fields] OR "serum s"[All Fields] OR "serumal"[All Fields])) OR "autologous conditioned serum"[All Fields] OR ("interleukin 1"[MeSH Terms] OR "interleukin 1"[All Fields] OR "interleukin 1"[All Fields])) AND ("lumbarised"[All Fields] OR "lumbarization"[All Fields] OR "lumbarized"[All Fields] OR "lumbars"[All Fields] OR "lumbosacral region"[MeSH Terms] OR ("lumbosacral"[All Fields] AND "region"[All Fields]) OR "lumbosacral region"[All Fields] OR "lumbar"[All Fields] OR ("cervic"[All Fields] OR "cervicals"[All Fields] OR "cervices"[All Fields] OR "neck"[MeSH Terms] OR "neck"[All Fields] OR "cervical"[All Fields] OR "uterine cervicitis"[MeSH Terms] OR ("uterine"[All Fields] AND "cervicitis"[All Fields]) OR "uterine cervicitis"[All Fields] OR "cervicitis"[All Fields]) OR "spin*"[All Fields] OR ("spine"[MeSH Terms] OR "spine"[All Fields] OR "vertebral"[All Fields] OR "vertebrals"[All Fields])) AND ("radicular"[All Fields] OR ("radiculopathy"[MeSH Terms] OR "radiculopathy"[All Fields] OR "radiculopathies"[All Fields]))	43
Scopus	(ALL (autologous AND serum OR "autologous conditioned serum" OR "interleukin 1") AND ALL (lumbar OR cervical OR spin* OR vertebral) AND ALL (radicular OR radiculopathy))	352
Embase	(autologous AND serum OR 'autologous conditioned serum' OR 'interleukin 1') AND (lumbar OR cervical OR spin* OR vertebral) AND (radicular OR radiculopathy)	81

4.2.2 Study Eligibility Criteria

The inclusion criteria included in the studies were as follows:

1. Randomised controlled trials, prospective or retrospective studies or case series that included adult patients more than 18 years with unilateral or bilateral lumbar or cervical radiculopathy.
2. The authors have reported the use of autologous conditioned serum, an interleukin 1 receptor antagonist.
3. The authors have reported one or more patient-reported outcome measures including Visual Analogue Scale for pain, Short Form of Health Survey-12 or Oswestry Disability Index.
4. A minimum clinical follow-up period of 3 months.

Exclusion criteria included abstracts, review articles, case reports, studies in animals and articles that did not report any relevant clinical data.

4.2.3 Data Extraction and Management

The study data was extracted in tabular form. Specifically, data pertaining to the these characteristics were collected: demographic details (author, country and year of publication, sample size, gender distribution, mean age group, median duration of symptoms, inclusion criteria, intervention) and outcome measures (VAS—Visual Analogue Scale, ODI—Oswestry Disability Index, SLRT—Straight Leg Raising Test, NDI—Neck Disability Index, NPDS—Neck Pain Disability Scale, PCS—Physical Component score of SF-12, MCS—Mental Component score of SF-12).

4.2.4 Methodological Quality Assessment of Included Studies

All studies were assessed to check the methodological quality of studies. Randomised studies were assessed using Jadad scale (Oremus et al. 2001). Aspects like random sequence generation, blinding of outcome assessments and other biases were assessed. Modified Jadad score greater than 4 was considered to be high-quality study. The Methodological Index for Non-randomised Studies (MINORS) was used to grade the risk of bias of non-comparative studies (Slim et al. 2003). Score of 13–15 was considered to have a low risk of bias and score of 12 or less was considered to have a high risk of bias. Each study was assessed for quality by two independent reviewers (P.S and A.G).

4.3 Results

4.3.1 Literature Search

The literature search revealed 476 articles. After removing the duplicates, 412 articles were examined by title and abstract. After exclusion by title/abstract, five articles were assessed by full text to consider for inclusion. One article included only abstract and was excluded (Moser et al. 2010). The remaining four studies were considered for the systematic review (Godek 2016; Goni et al. 2015; Ravi Kumar et al. 2015; Becker et al. 2007). The literature selection process is shown in Fig. 4.1.

4.3.2 Study Characteristics

All the studies evaluated the effect of autologous concentrated serum on radiculopathy. Out of the four studies, two were randomised controlled trials (level 1) and two studies were prospective non-comparative studies (level 4). One study compared the efficacy between ACS and methyl prednisolone (MPS) and one study compared ACS with two triamcinolone concentrations. Three studies evaluated the effect of the interleukin 1 receptor antagonist on lumbar radiculopathy while one study evaluated the effect on cervical radiculopathy. A total of 107 patients received ACS for treatment.

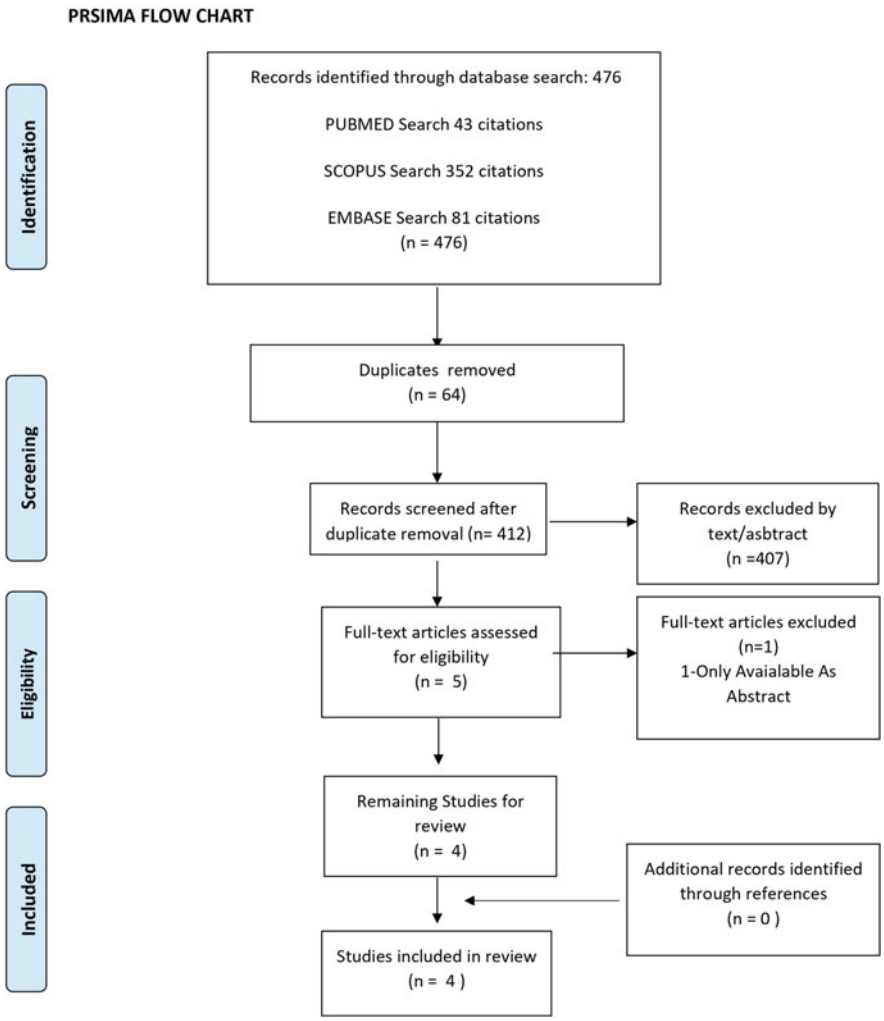


Fig. 4.1 PRISMA flow diagram for study selection

4.3.3 Demographic Variables

All the studies had a predominance of male patients, except Ravi Kumar et al. who have not detailed about gender distribution (Ravi Kumar et al. 2015). The patients were adults who had symptoms of unilateral radiculopathy for at least 6 weeks. The included patients had both clinical and radiographic signs of disc herniation in the spine causing the symptoms. The mean age of patients in the studies ranged from 37.15 to 53.9. The dose of ACS used was 2–4 mL injection of ACS (Table 4.2).

Table 4.2 Demographics and details of selected studies for the systematic review

Author	Country and year of publication	Sample size	Gender distribution	Mean age group (in years)	Median duration of symptoms	Inclusion criteria	Intervention (injection to affected intervertebral foramen)	Outcome measures	Follow-up (after first injection)
Godek et al.	Poland, 2016	15	Male: 9 (60%) Female: 6 (40%)	38.9 ± 8.9 (27–54)	16 weeks (IQ 5.5–42-2 weeks)	• Age > 18 years • Clinical + radiographic signs of single level nucleus pulposus herniation in the lumbar spine • Unilateral lumbar radiculopathy	3–4 mL injection of ACS	• VAS • ODI • One leg standing test • SLRT	• 1 month • 3 months
Goni et al.	India, 2015	40	Male: 23 (57.5%) Female: 17 (42.5%)	44.52	13.8 months (ACS 14.5 months (methylprednisolone)	• Age: 30–60 years • Unilateral cervical radiculopathy >6 weeks duration • Pain with VAS > 7/10 • Radiological and clinical signs of nerve root nerve root involvement	2–3 mL injection of • ACS (study) • Methyl prednisolone (control)	• VAS • NPDS • NDI • SF-12	• 3 weeks • 3 months • 6 months

(continued)

Table 4.2 (continued)

Author	Country and year of publication	Sample size	Gender distribution	Mean age group (in years)	Median duration of symptoms	Inclusion criteria	Intervention (injection to affected intervertebral foramen)	Outcome measures	Follow-up (after first injection)
Ravi Kumar et al.	India, 2015	20		37.15		• Age: 30–60 years • Unilateral lumbar radiculopathy >6 weeks duration • Clinical (positive SLRT) and radiological signs of lumbar nerve root involvement	2 mL injection of ACS	• VAS • ODI • SLRT • SF-12	• 3 weeks • 3 months • 6 months
Becker et al.	Germany, 2007	84	Male: 52 (61.9%) Female: 32 (28.1%)	53.9 (29–81)		• Unilateral lumbar radiculopathy • Pain >6 weeks duration • Radiology showing nucleus pulpous degeneration	• ACS • 5 mg triamcinolone • 10 mg triamcinolone	• VAS • ODI	• 6 weeks • 10 weeks • 22 weeks

ACS autologous conditioned serum, *VAS* Visual Analogue Scale, *ODI* Oswestry Disability Index, *SLRT* Straight Leg Raising Test, *NDI* Neck Disability Index, *NPDS* Neck Pain Disability Scale, *SF-12* 12 item short form survey

4.3.4 Methodological Quality Assessment

Both the RCTs were well-conducted trials and have properly described randomisation method. Overall, both the RCTs showed high methodological quality as evaluated by the Jadad scale (Oremus et al. 2001). On evaluation by the MINORS criteria, the two non-comparative prospective studies have achieved a grade of C (score of 12 or less), indicating a high risk of bias and moderate study quality. Overall the studies ranged from moderate to high quality of studies (Tables 4.3 and 4.4).

4.3.5 Medication Use and Injection Protocol

Treatment injection: The intervention included use of 2–4 mL of autologous conditioned serum. The ACS was prepared according to the technique described by Meijer et al. (2003). Godek et al. utilised ultrasound guidance as a means to inject the ACS, while the other three studies have utilised fluoroscopic imaging guidance to inject the ACS (Godek 2016). The injections were administered by epidural perineural technique in all. The injection was repeated weekly, up to a maximum of three doses overall, depending on the pain intolerance.

Control injection: One RCT divided the patients into two groups, in which a similar amount of methylprednisolone was used as control (Becker et al. 2007). In the other RCT, three groups were used in which 5 mg and 10 mg of triamcinolone were used as control (Goni et al. 2015).

4.3.6 Patient-Reported Clinical Outcomes

1. ***Visual Analogue Scale*:** All the four studies reported VAS pain as an outcome measure. Ravi et al. reported a significant change in VAS at each follow-up from pre-injection up to the third follow-up ($p < 0.001$) (Ravi Kumar et al. 2015). Godek et al. have also reported a statistically significant reduction in VAS at first and second follow-up (Godek 2016). Becker et al. found a significant change in VAS score within the treatment group from pre-injection to final follow-up ($p < 0.001$); they also found a statistically significant difference ($p = 0.046$) when compared to triamcinolone 5 mg group (but not 10 mg group) at the final follow-up (Becker et al. 2007). Goni et al. also found a good improvement of 73.2% over the baseline score in VAS at the follow-up time of 6 months (Goni et al. 2015) (Table 4.5).
2. ***Oswestry Disability Index*:** Three studies have reported Oswestry Disability Index in the patients (Godek 2016; Ravi Kumar et al. 2015; Becker et al. 2007). Ravi et al. reported the ODI score has significantly improved from pre-injection to the final follow-up ($p < 0.001$) and also in between the follow-ups ($p = 0.001$). Godek et al. reported a baseline ODI score of 39.2%, which improved to 20.9% and 14.7% at first month and third month, respectively. As per

Table 4.3 Quality assessment of the study by Modified Jadad scores

Corresponding author	Was the research described as randomised?	Was the approach of randomisation appropriate?	Was the research described as blinding?	Was the approach of blinding appropriate?	Was there a presentation of withdrawals and dropouts?	Was there a presentation of the inclusion/ exclusion criteria?	Was the approach used to assess adverse effects described?	Was the approach of statistical analysis described?	Total
Goni et al.	1	1	1	1	0	1	1	1	**7**
Becker et al.	1	1	1	0	1	1	1	1	**7**

Table 4.4 Quality assessment of the included studies by Individual MINORS score

	Clearly stated aim	Inclusion of consecutive patients	Prospective data collection	Endpoints appropriate to study aim	Unbiased assessment of study endpoint	Follow-up period appropriate to study aim	<5% lost to follow-up	Prospective calculation of study size	Adequate control group	Contemporary groups	Baseline equivalence of groups	Adequate statistical analyses	Total
Ravi Kumar et al.	2	2	2	2	0	2	2	0	NA	NA	NA	NA	12/16
Goni et al.	2	2	1	2	0	2	2	0	NA	NA	NA	NA	11/16

Table 4.5 Base line and follow up outcome scores of the included studies

	Baseline						
Author	VAS	ODI	PCS	MCS	SLRT	NDI	NPDS
Godek et al.	55	39.2%			60.00		
Goni et al.	• ACS-71 • MPS-69		• ACS-27.35 • MPS-28.22	• ACS-36.22 • MPS-36.53		• ACS-62.3 • MPS-64.4	• ACS-70.70 • MPS-70.05
Ravi Kumar et al.	69.5	27.9%	27.25	36.59	42.00		
Becker et al.	• ACS-78 • Triamcinolone 5 mg-82 • Triamcinolone 10 mg-85	• ACS-22% • Triamcinolone 5 mg-20.6% • Triamcinolone 10 mg-19.4%					

Follow-up						
	VAS (mean difference)			ODI/NDI		
Author	I	II	III	I	II	III
Godek et al.	44 (20%)	36(34.4%)		20.9%	14.7%	
Goni et al.	• ACS-35 • MPS-22	• ACS-24 • MPS-25.5	• ACS-19 • MPS-27.5	• NDI-ACS-35.00% • NDI-MPS-19.30%	• NDI-ACS-20.00% • NDI-MPS-25.10%	• NDI-ACS-15.9% • NDI-MPS-30.40%
Ravi Kumar et al.	36.5	22.5	20	14.95%	10.5%	8.5%
Becker et al.			• ACS-23.3±24.8 • Triamcinolone 5 mg-36.8±28.3 • Triamcinolone 10 mg-32.6±28.2	• ACS-13.8% • Triamcinolone 5 mg-12.1% • Triamcinolone 10 mg-11%	• ACS-11.2% • Triamcinolone 5 mg-12.4% • Triamcinolone 10 mg-11%	• ACS-11.7% • Triamcinolone 5 mg-11.1% • Triamcinolone 10 mg-11.4%

(continued)

Table 4.5 (continued)

Follow-up									
	SF-12 (PCS/MCS)			SLRT			NPDS		
Author	I	II	III	I	II	III	I	II	III
Godek et al.				75	77				
Goni et al.	• ACS-39.47/43.09 • MPS-48.70/45.76	• ACS-46.60/45.17 • MPS-45.90/43.16	• ACS-49.08/47.12 • MPS-44.39/42.42				• ACS-34.05 • MPS-23.85	• ACS-24.70 • MPS-27.95	• ACS-18.55 • MPS-31.1
Ravi Kumar et al.	40.08/43.46	47.59/45.79	49.32/47.51	69	74	76			
Becker et al.									

ACS Autologous conditioned serum, *VAS* Visual Analogue Scale, *ODI* Oswestry Disability Index, *SLRT* Straight Leg Raising Test, *NDI* Neck Disability Index, *NPDS* Neck Pain Disability Scale, *MPS* Methyl Prednisolone, *PCS* Physical Component score of SF-12, *MCS* Mental Component score of SF-12

Becker et al., the within-group analysis of ODI score in the ACS group improved significantly ($p < 0.001$); however, there was no statistically significant difference between the ACS group and the two triamcinolone groups.

3. ***Straight Leg Raising Test*:** Two studies have reported changes in SLRT scores (Godek 2016; Ravi Kumar et al. 2015). Ravi et al. reported a significant change from pre-injection to first, second and final follow-up ($p < 0.001$); however, the changes in between the follow-ups were not significant ($p = 0.399$ for first to second follow-up and $p = 0.115$ for second to third follow-up). Godek et al. also stated that SLR test showed a higher degree of leg raise without pain.
4. ***SF-12:*** Two studies reported PCS and MCS components of SF-12 (Goni et al. 2015; Ravi Kumar et al. 2015). Ravi et al. reported a significant difference between pre-injection and first, second and third follow-up ($p < 0.001$) and also between first and second follow-up ($p = 0.001$) and between second and third follow-up ($p = 0.001$). The MCS has also improved significantly from the pre-injection time to all the three follow-ups ($p < 0.001$); however, there was no significant change in MCS in-between the follow-ups. Goni et al. reported improvement in PCS from 27.35 (pre-injection) to 49.08 at the final follow-up, an increase of 79.45% and the MCS improved by 30.09% at the final follow-up.
5. ***Neck Pain Disability Scale and Neck Disability Index:*** Only Goni et al. reported these two outcomes as they have evaluated ACS in cervical radiculopathy (Goni et al. 2015). The mean NPDS score showed a decrease of 73.76% from the pre-injection to the final follow-up (in comparison to 55.6% in the MPS group). The NDI showed a decrease of 74.47% compared with 52.80% in the MPS group from pre-injection to the final follow-up.

4.4 Discussion

Four studies were reviewed, and all of the studies concluded that epidural perineural injection autologous conditioned serum reduced pain scores (VAS, NPDS) and improved functional scores (ODI, SF-12 and NPDS), as compared to placebo and other conventional therapeutic modalities like steroids and analgesic-anaesthetic-steroid cocktail. ACS is a rich source of autologous IL-1Ra, derived from venous blood of the patient and incubated with medical-grade glass beads for 24 h, and the IL-1Ra and other anti-inflammatory cytokines like TGF-β, FGF and PDGF are thought to be partially from cell break down and partially generated de novo from monocytes as proven by cycloheximide inhibition (Ajrawat et al. 2019). IL-1 and TNF-α are the primary inflammatory cytokines implicated in multiple inflammatory arthritis and age-related degenerative processes (Wehling et al. 2007). Targeted therapy to these cytokines may help arrest the progress of inflammation-related damage and growth factors may even aid regeneration. Therefore, ACS is a promising prospect for future use in non-operative therapy of radiculopathy. Low back ache and neck pain due to radiculopathy are some of the most common cases faced by the orthopaedic surgeons in an outpatient setting. The use of autologous IL-1Ra seems to be effective and longer lasting than synthetic IL-1Ra (Anakinra),

and more level-1 studies are needed to ascertain the efficacy and benefits. Also cost benefit analysis is needed for main stream usage.

Use of ACS in osteoarthritis of knee has already been extensively studied. Baltzer et al. compared the use of intra-articular ACS, with intra-articular hyaluronic acid and saline in 376 patients, and concluded that ACS significantly reduced Western Ontario and McMaster Universities osteoarthritis (WOMAC) index at all time intervals (3 months and 6 months) as compared to the other groups ($p < 0.001$ for all comparisons). VAS ratings were also lowest in the ACS group ($p < 0.001$) (Baltzer et al. 2009). Even at the end of 2 years, WOMAC and VAS were significantly lesser ($p < 0.001$) in the ACS group (Baltzer et al. 2009). ACS has been found to be superior to other orthobiologics in common use like platelet-rich plasma (PRP). In a study conducted of 123 women with moderate osteoarthritis, WOMAC and VAS in both groups (PRP and ACS) were comparable in patients with subclinical early synovitis at the end of 3 months. However, in patients with moderate clinically significant synovitis, ACS was found to be superior to PRP at the end of 3 months with significant improvement in WOMAC and VAS ($p < 0.001$ for both comparisons) (Shirokova et al. 2017).

ACS has also been used in muscle and ligament injuries. It has been used after anterior cruciate ligament (ACL) reconstruction to reduce bone tunnel widening. Post-operative inflammation leading to increased intra-articular IL-1β levels, which in turn increases osteoclastic activity, has been implicated in bone tunnel widening, producing less than satisfactory results. Daraboset al. conducted a study in 62 patients and found that intra-articular ACS injection significantly reduced bone tunnel widening ($p < 0.05$) and synovial IL-1β levels. The ACS group had better WOMAC scores at 1-year follow-up ($p = 0.047$) (Darabos et al. 2011).

Carpenter et al. conducted a pilot study in sportsmen with muscle strains and found that ACS injection shortened the time to recovery, showed early improvement in MRI findings and led to early return to sports (Wright-Carpenter et al. 2004a). Carpenter et al. also studied the effect of ACS on muscle regeneration in a rat muscle contusion model and found that due to high concentrations of FGF-2 and TGF β in ACS, it increased the proliferation of satellite cells; thus, ACS-treated muscles had a larger percentage of large regenerating myofibres, showing that ACS potentially aids muscle healing (Wright-Carpenter et al. 2004b).

Orthobiologics are the emerging new therapeutics, like PRP for osteoarthritis of the knee and BMP (bone morphogenetic protein) in the spinal fusion and non-unions; in a similar manner, the anti-inflammatory effects of ACS can be harnessed and further research needs to be carried out on the use of ACS in other inflammatory arthritis like rheumatoid arthritis, gout and ankylosing spondylitis and also in injury-related inflammation, where also IL-1 is a key cytokine in the inflammatory process. ACS has already been effective in multiple other orthopaedic problems, and it has been used effectively in chronic Achilles tendinopathy. It has also been effective in animal muscle contusion models (Genç et al. 2018; von Wehren et al. 2019). Surely, ACS is a promising new therapeutic modality in both lumbar and cervical radiculopathy, and further studies can strengthen the present evidence regarding its efficacy and safety profile.

References

Ajrawat P, Dwyer T, Chahal J (2019) Autologous interleukin 1 receptor antagonist blood-derived products for knee osteoarthritis: a systematic review. Arthroscopy 35:2211–2221

Baltzer AWA, Moser C, Jansen SA, Krauspe R (2009) Autologous conditioned serum (Orthokine) is an effective treatment for knee osteoarthritis. Osteoarthr Cartil 17:152–160

Becker C, Heidersdorf S, Drewlo S, De Rodriguez SZ, Krämer J, Willburger RE (2007) Efficacy of epidural perineural injections with autologous conditioned serum for lumbar radicular compression: an investigator-initiated, prospective, double-blind, reference-controlled study. Spine 32: 1803–1808

Brown CA, Toth AP, Magnussen B (2010) Clinical benefits of intra-articular anakinra for arthrofibrosis. Orthopedics [Internet] 33(12):877. SLACK Incorporated; [Cited 2020 Oct 11]. https://www.healio.com/orthopedics/knee/journals/ortho/2010-12-33-12/{8e445a8f-8d89-44fe-bb21-40541d751fcf}/clinical-benefits-of-intra-articular-anakinra-for-arthrofibrosis

Chevalier X, Goupille P, Beaulieu AD, Burch FX, Bensen WG, Conrozier T et al (2009) Intraarticular injection of anakinra in osteoarthritis of the knee: a multicenter, randomized, double-blind, placebo-controlled study. Arthritis Care Res 61:344–352

Cohen SP (2015) Epidemiology, diagnosis, and treatment of neck pain. Mayo Clin Proc 90:284–299

Daniels J, Binch AAL, Maitre CLL (2017) Inhibiting IL-1 signaling pathways to inhibit catabolic processes in disc degeneration. J Orthop Res 35:74–85

Darabos N, Haspl M, Moser C, Darabos A, Bartolek D, Groenemeyer D (2011) Intraarticular application of autologous conditioned serum (ACS) reduces bone tunnel widening after ACL reconstructive surgery in a randomized controlled trial. Knee Surg Sports Traumatol Arthrosc 19:36–46

Genç E, Beytemur O, Yuksel S, Eren Y, Çağlar A, Küçükyıldırım BO et al (2018) Investigation of the biomechanical and histopathological effects of autologous conditioned serum on healing of Achilles tendon. Acta Orthop Traumatol Turc 52:226–231

Godek P (2016) Use of autologous serum in treatment of lumbar radiculopathy pain. Pilot study. Ortop Traumatol Rehabil 18:11–20. Agencja Wydawnicza Medsportpress

Goni VG, Singh Jhala S, Gopinathan NR, Behera P, Batra YK, Arjun RHH et al (2015) Efficacy of epidural perineural injection of autologous conditioned serum in unilateral cervical radiculopathy. Spine 40:E915–E921

Hoy D, Bain C, Williams G, March L, Brooks P, Blyth F et al (2012) A systematic review of the global prevalence of low back pain. Arthritis Rheum 64:2028–2037

Johnson ZI, Schoepflin ZR, Choi H, Shapiro IM, Risbud MV (2015) Disc in flames: roles of TNF-α and IL-1β in intervertebral disc degeneration. Eur Cell Mater 30:104–117

Mansfield M, Smith T, Spahr N, Thacker M (2020) Cervical spine radiculopathy epidemiology: a systematic review. Musculoskeletal Care [Internet]. [Cited 2020 Oct 10]; n/a. https://www.onlinelibrary.wiley.com/doi/abs/10.1002/msc.1498

Meijer H, Reinecke J, Becker C, Tholen G, Wehling P (2003) The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. Inflamm Res 52:404–407

Moher D, Liberati A, Tetzlaff J, Altman DG (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 6:e1000097

Moser C, Groenemeyer D, Wehling P (2010) Efficacy of epidural injections with autologous conditioned serum (ACS) for lumbar radicular compression. Eur Spine J 19:1404

Oremus M, Wolfson C, Perrault A, Demers L, Momoli F, Moride Y (2001) Interrater reliability of the modified Jadad quality scale for systematic reviews of Alzheimer's disease drug trials. Dement Geriatr Cogn Disord 12:232–236

Ravi Kumar HS, Goni VG, Batra YK (2015) Autologous conditioned serum as a novel alternative option in the treatment of unilateral lumbar radiculopathy: a prospective study. Asian Spine J 9: 916–922. Korean Society of Spine Surgery

Shirokova K, Noskov S, Shirokova L (2017) Comparison of clinical efficacy of platelet-rich plasma and autologous conditioned serum treatment in patients with osteoarthritis of the knee. Osteoarthr Cartil 25:S438. Elsevier

Slim K, Nini E, Forestier D, Kwiatkowski F, Panis Y, Chipponi J (2003) Methodological index for non-randomized studies (minors): development and validation of a new instrument. ANZ J Surg 73:712–716

von Wehren L, Pokorny K, Blanke F, Sailer J, Majewski M (2019) Injection with autologous conditioned serum has better clinical results than eccentric training for chronic Achilles tendinopathy. Knee Surg Sports Traumatol Arthrosc 27:2744–2753

Wehling P, Moser C, Frisbie D, McIlwraith CW, Kawcak CE, Krauspe R et al (2007) Autologous conditioned serum in the treatment of orthopedic diseases: the orthokine therapy. BioDrugs 21: 323–332

Wright-Carpenter T, Klein P, Schäferhoff P, Appell HJ, Mir LM, Wehling P (2004a) Treatment of muscle injuries by local administration of autologous conditioned serum: a pilot study on sportsmen with muscle strains. Int J Sports Med 25:588–593

Wright-Carpenter T, Opolon P, Appell HJ, Meijer H, Wehling P, Mir LM (2004b) Treatment of muscle injuries by local administration of autologous conditioned serum: animal experiments using a muscle contusion model. Int J Sports Med 25:582–587

Bench-to-Bedside Research in Ophthalmology

5

Amod Gupta

Abstract

Dawn of the twenty-first century has seen a concerted focus on bench research that could be translated into therapeutic interventions in hitherto blinding eye diseases that either did not have any treatment or had at best a very limited successful outcome. This focused approach in laboratory research, aided largely by an exponential growth in the biotechnology tools, has led to a better understanding of the highly complex cell biology and the pathophysiological disease pathways and the discovery of the potential therapeutic targets for interventions. Inherited retinal disorders, the commonest example of which the retinitis pigmentosa affects millions of patients worldwide, have its onset in early childhood and make the affected blind in the prime of youth. Discovery of the affected genes and extensive research for safe and effective vectors for gene transfer led to among the first FDA-approved gene therapy with Luxturna and the beneficial results sustainable up to 4 years of the follow-up currently available. For the patients who do not have any viable photoreceptors or have a polygenic disease, a breakthrough has been achieved in optogenetics with the successful expression of an opsin gene in bipolar cells of the retina which are the second downstream neuron in the visual pathways. The initial results of its successful use in humans were reported recently. Several controlled clinical trials are underway for gene therapy in age-related macular degeneration which affects nearly 200 million people in the world both for the dry and the wet or neovascular degeneration (nAMD). Discovery of the vascular endothelial growth factor (VEGF) in 1989 and development of its antibodies soon after have led to a revolutionary change in the management of blinding retinal diseases like nAMD, diabetic macular

A. Gupta (✉)
Advanced Eye Centre, Post Graduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_5

oedema, proliferative diabetic retinopathy and the retinal vascular occlusions. The standard of care has shifted from the destructive laser photocoagulation to the use of intraocular injections, currently the most frequent interventional procedure performed among all the procedures done for the human diseases across the spectrum. Glaucoma is another focus of translational research and is now recognized as a neurodegenerative disorder. Several strategies are being tested to treat it with a variety of neurotrophic factors. Cell-based therapies have seen the first human photoreceptor transplant in Japan from a donor 3-D retina organoid grown in the lab. Human embryonic stem cells are being tested in several clinical trials to repopulate the retinal pigment epithelial layer, one of the most vital layers that sustain photoreceptors. Discovery of the polymerase chain reaction to generate billions of copies of DNA not only revolutionized the field of cell biology but also provided a highly sensitive tool to diagnose intraocular infections with hitherto difficult to diagnose fastidious organisms that cause ocular infections. Exponential growth in the understanding of the inflammatory pathways has found new targets for the treatment of inflammatory eye diseases with safe and effective biological antibodies that are being increasingly used in the clinics all over the world to save patients from going blind.

Keywords

nAMD · Dry AMD · Geographic atrophy · Diabetic retinopathy · Diabetic macular oedema · Proliferative diabetic retinopathy · Glaucoma · PCR · Vascular endothelial growth factor · VEGF · Avastin · Lucentis · Eylea · Retinitis pigmentosa

5.1 Introduction

Eyes, often dismissed as mere 1-inch balls that perceive light and formed vision, are a unique microcosm of the human body that have the representation of all the major human body tissues such as the epithelial, the connective, the neural and the muscles. The tissues in the eye, however, get highly specialized such as the transparent cornea—connective tissue lined by epithelium and an autofocus transparent crystalline lens of epithelial origin that besides transmitting light in the visible spectrum also refract it to focus on a multilayered neural retina at the back of the eye (Figs. 5.1 and 5.2). The white of the eye, the sclera, is the outer coat of the eyeball made of collagen, a structural connective tissue that provides rigidity and the form of the ball. It shares properties with the cartilage of the various body joints and gets affected in some of the diseases that target the joints. The specialized humours, the constantly circulating aqueous and the vitreous that fill and keep the eyeball pressurized, also provide nutrition to the ocular tissues. Like the central nervous system and the testes, the eyes are also an immune-privileged site. They have a unique blood supply that boasts of strict barriers to prevent the entry of undesirable substances into the eye and in that sense share some properties with the blood supply of the brain.

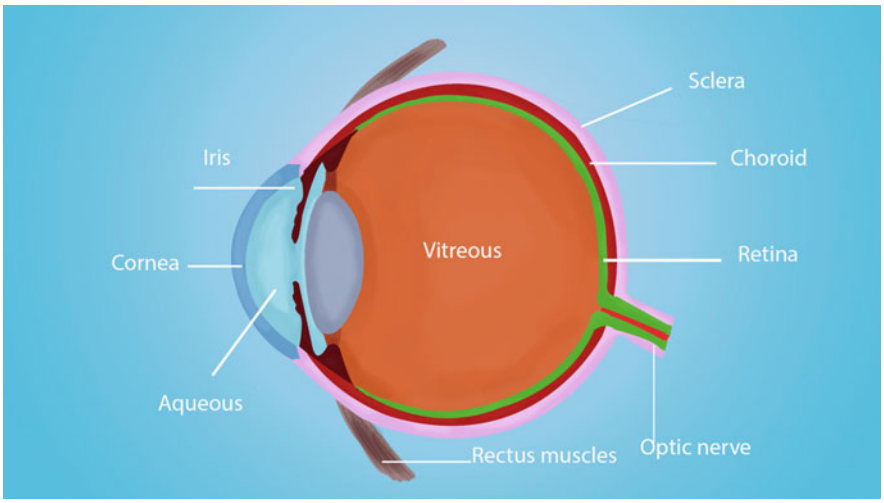


Fig. 5.1 Schematic cross-section of the eyeball to show various layers and general scheme of structures (Graphics: Kritika Thakur)

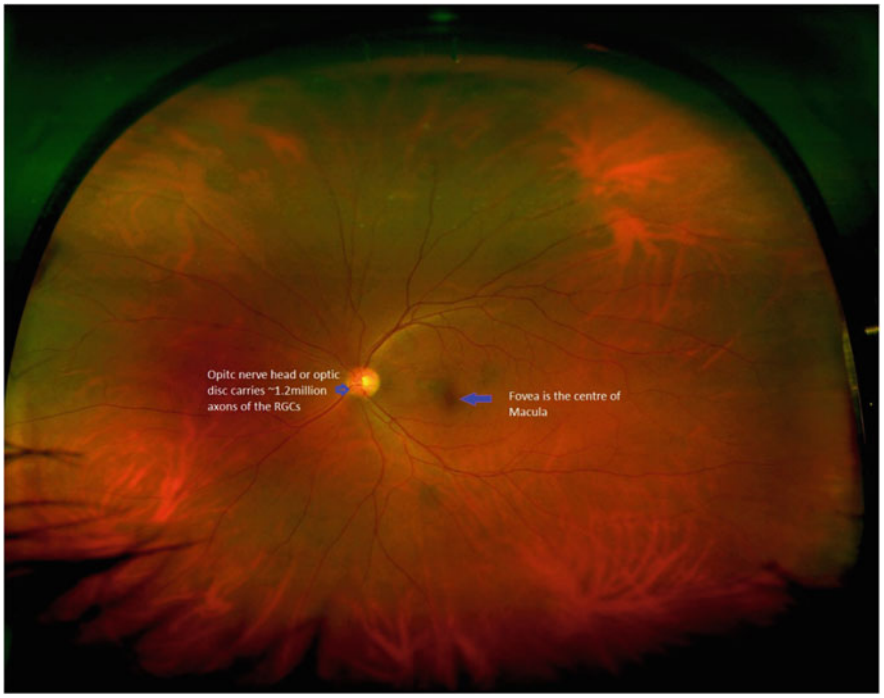


Fig. 5.2 An ultra-wide view, covering nearly 82% (200°) of the retina in the left eye

5.1.1 Opportunities for Translational Research in Ophthalmology

Eyes provide a unique opportunity to study various elements of vision, physiology of vision, optics of light, complexities of light transmission through ocular tissues, laser-tissue interactions, biomaterials, intraocular prosthetics and development of optomechanical instruments to study the structures and functioning of the eye in health and disease that dominated the latter half of the nineteenth and the entire twentieth century. In the last 20–30 years, there has been an exponential growth in bench research in the fields of molecular and cellular biology, immunology, mechanisms of ageing and retinal degeneration, genetics, genomics and proteomics, stem cell, prosthetic vision and development of biomarkers. The focus of research has shifted to demystify the pathways of disease and develop therapeutic interventions targeting at the root cause of the disease. Most of the translational research in ophthalmology is carried out in collaboration with basic science disciplines in the university settings and has led to revolutionary applications in the clinical practice.

5.1.2 Defining the Bench-to-Bedside Research

To improve the health of the people, 'effective translation of the new knowledge, mechanisms and techniques generated by advances in basic science research into new approaches for prevention, diagnosis and treatment of the disease' is essential (Fontanarosa and DeAngelis 2002). A relatively new discipline, 'bench-to-bedside' research or 'the translational research' simply means the application of the scientific research or discoveries made in the laboratory to the patients in the clinic and the community. The idea of having research laboratories next to a clinic to unravel the mysteries of disease was first mooted by William Osler, the co-founder of the Johns Hopkins Hospital in Baltimore and is more than 100 years old. Despite the phenomenal progress in basic science research, its application was painfully slow in the initial decades as the scientists and clinicians very often worked in isolation. Contopoulos-Ioannidis et al. (2003) identified 101 basic science articles published in six major basic science journals from 1979 to 1983, which had clearly defined preventive or therapeutic applications, of which 19 arrived at a positive outcome in randomized controlled trials but just five of these basic science technologies got licensed for clinical use. While the start of the MD-PhD programmes has been a major catalyst, it is only in recent years that exponential growth is occurring in this paradigm (Ryan et al. 2002). The National Institutes of Health (NIH) has declared translational research a priority area and besides announcing a Clinical and Translational Science Award (CTSA) in 2006, it has created centres for translational research in its institutes (Woolf 2008). Although this field is highly exciting, at the same time, it is full of challenges throughout the life of a scientist. If a promising technology fails to reach a published human study within 10–12 years, it was unlikely to be tested in humans (Contopoulos-Ioannidis et al. 2003). The scientists should collaborate with industry in their basic research as it increases the chances of

its reaching a human trial stage by nearly eight times (Contopoulos-Ioannidis et al. 2003).

5.1.3 Expanding Horizons of Translational Research

More recently, a consensus is emerging that the scope and definition of translational research need to expand to include at least five distinct phases (T0 to T4); in T1, the typical bench-to-bedside research is developing basic research ideas; phase 1 and 2 involve trials in humans; it also includes genomics, animal studies, drug development, etc.; phase T2 includes the phase 3 clinical trials to establish the efficacy of new interventions in humans and developing clinical guidelines; in T3, the focus is on the implementation and dissemination of research and in T4 to study the outcomes in diverse populations and off-target adverse effects as a result of unintended modulation of other targets. The T0 phase involves taking the research ideas back to the bench (Zarbin 2020; Fort et al. 2017). This chapter will focus on T1 and T2 stages of translational research.

5.1.4 Challenges for the Ophthalmologists on Starting Translational Research

The basic scientists must collaborate with the clinicians to understand the felt need of patients and the community. The most effective translational research comes from asking simple but right questions in the clinic. A simple observation in the clinic, that superficial punctate keratitis occurred in the lower part of the cornea due to incomplete blinking, led to more than 40 years of research from bed to bench and back to bed in dry eye syndromes (Abelson et al. 2020). The ophthalmologists often work in isolation and even if they have the right questions requiring exploration in the laboratories they are often not aware of the emerging concepts and the new developments occurring in the laboratory sciences. Complexities of the experimental and biological systems and so also the language of communication used by the basic scientists are often too alien for the clinicians. One way to overcome this challenge is to expose the basic scientists to clinical problems and the clinicians to the basic sciences (Knox 2012). While funding and manpower resources are a major impediment in carrying out translational research, regulatory and ethical issues have to be overcome before the fruits of translational research can reach the clinics and the community (Chawla 2018). Beginning 2021, the Association for Research in Vision and Ophthalmology (ARVO) will initiate a new programme to encourage and educate vision scientists on converting their translational ideas into clinical products [https://www.arvo.org/meetings/bench-to-bedside-meeting/].

5.2 Application of Translational Research in Ophthalmology

5.2.1 Neuroprotection and Neuroregeneration

In recent years, 'neuroprotection' and 'neuroregeneration' have become the fastest-growing translational research fields in ophthalmology. The retina, the neurosensory layer of the eye, is composed of postmitotic cells, and once these cells degenerate or undergo apoptosis, these neurons cannot be revived (Figs. 5.3 and 5.4). Basic science research in the field of cell biology has elucidated several pathways that participate in cell survival and apoptosis. This has led to the identification of several potential targets that can be utilized for promoting survival and regeneration of neurons. The challenge is to prevent the disease by reversing the pathological state of the neurons into the physiological state before these neurons undergo permanent necrosis. RGC axonal injuries commonly follow an ischaemic or traumatic insult leading to retrograde apoptosis of the RGCs. There are no progenitor cells in the retina to replace the dead RGCs, and therefore the challenge remains in keeping the RGC healthy or reverse the state of its health. All attempts to regrow the axons have not met with success as the glial scars present an impediment for the growth of axons beyond the site of injury, and it has also not been possible to regenerate the myelin sheath of the axons. Therefore, once optic atrophy occurs, it cannot be reversed. The subject has been exhaustively reviewed in 2020 (Boia et al. 2020; Gokoffski et al. 2020). Moreover, since many of the retinal degenerative diseases are multifactorial,

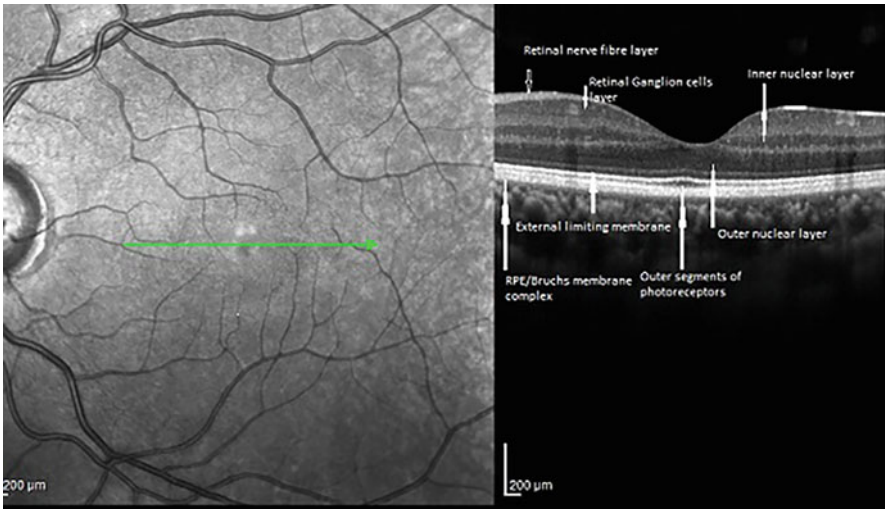


Fig. 5.3 Spectral-domain optical coherence tomography (OCT) horizontal line scan (green line in the left image) through the centre of fovea in the left eye of a normal person shows multilayered ultrastructure of the neurosensory retina, retinal pigment epithelium and choroid. Note that the outer nuclear layer in the foveal centre, representing the nuclei of photoreceptors, is extending throughout the layers of the retina. In this area this consists of very high density of cone photoreceptors

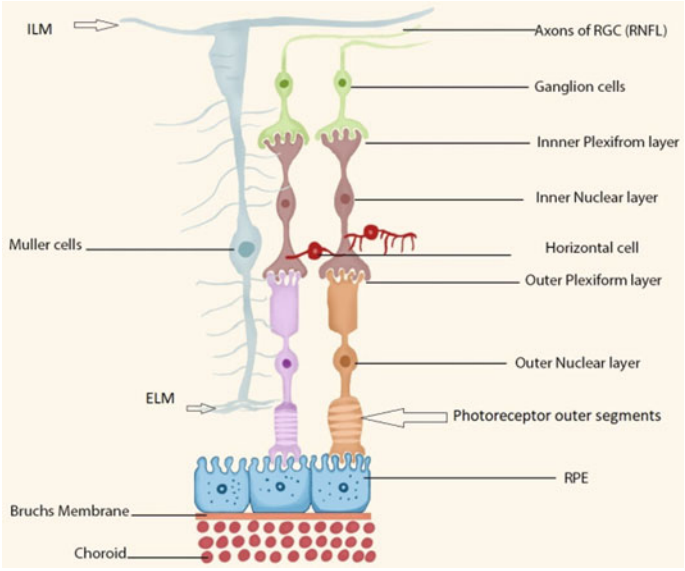


Fig. 5.4 Highly schematic sketch to show arrangement of the main cell bodies and their fibres in the retina. Outer nuclear layer represents the cell bodies of photoreceptors, the cones and rods, the inner nuclear cells represent the cell bodies of bipolar, amacrine, the horizontal and the Muller cells. Axons of the retinal ganglion cells (RGC) constitute the retinal nerve fibre layer (RNFL). Footplates of the Muller cells form the internal limiting membrane (ILM) in front of the axons of the RGC. Muller cells are macroglia cells and have intimate contact with the retinal capillaries and the neuronal cell bodies and control the microenvironment through formation of neuro-glio-vascular units. Outer limiting membrane (OLM) is formed by the contact of Muller cell and the junction of the photoreceptor inner and outer segments (Graphics: Kritika Thakur)

correction of any single factor may not be entirely successful (Payne et al. 2013). Very often the promising results of the therapeutic targeting in preclinical research in knockout animals fail to replicate when tested in human clinical trials.

5.2.2 Retinal Degeneration

Glaucoma is an asymptomatic neurodegenerative disorder affecting nearly 80 million people worldwide and causes irreversible blindness due to progressive atrophy of the retinal nerve fibres (approximately 1–1.2 million in each normal eye) and manifests clinically as a progressive enlargement of the cup in the optic nerve head, the optic disc cupping, with the corresponding loss of visual field. The retinal nerve fibres are the axons of the retinal ganglion cells (Fig. 5.5), the third neuron in the visual pathway that communicates visual signals from the retinal photoreceptors (first neuron) via bipolar cells (second neuron) to the lateral geniculate nucleus (LGN) in the midbrain, which further communicates with the visual centre in the occipital

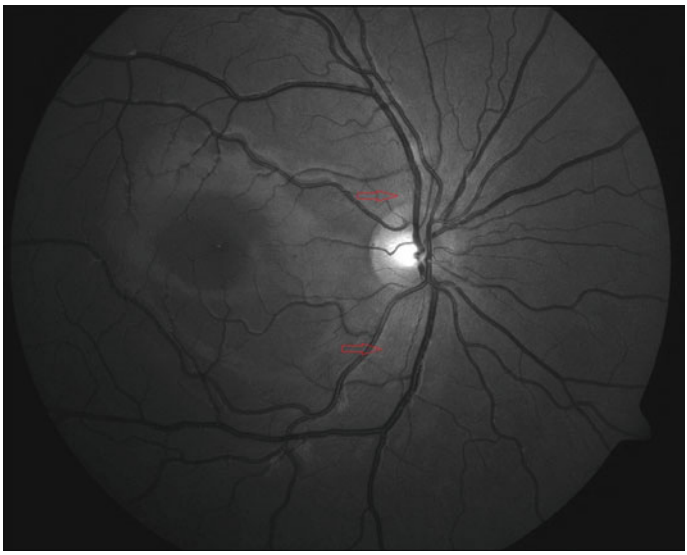


Fig. 5.5 A red-free image of the right eye shows (arrows) finely striated bundles of the axons of the retinal ganglion cells (RGCs). These axons also called the retinal nerve fibre layer (RNFL) become visible as the fibres converge to enter the optic nerve head called the optic disc

cortex. Till recently, all therapies (medicines, lasers and incisional surgery) were directed towards lowering the intraocular pressure, the only known major risk factor for the progression of the disease, yet a little less than half of the patients despite control of the pressure continued to suffer progression of the disease. Primate models of glaucoma and pathology of human glaucoma have revealed atrophy of the magnocellular and parvocellular RGCs and the loss of the corresponding layers in the LGN, making it a neurodegenerative disease, thus bringing in the role of neurotrophic factors such as the ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BNDF) and the pigment epithelium-derived factor (PEDF) (Tsai 2013).

5.2.2.1 Basic of Neuroprotection

There are several potential targets for glaucoma treatment based on the understanding of the mechanisms of the cell survival and death of RGCs. For a long time, G-protein-coupled receptor (GPCR) proteins have been targets for drug development for the control of glaucoma. B-blockers such as timolol and prostaglandin analogues such as latanoprost, travoprost and tafluprost work through their interaction with the GPCR proteins (He et al. 2018). The biology of the neuronal cells has been recently reviewed (He et al. 2018). Cell death can be triggered by several caspase-dependent or independent mechanisms. Briefly, the *N*-methyl-D-aspartate (NMDA), a protein on the neuronal cells, is a receptor for one of the most important neurotransmitters, the glutamate, and provides a glutamate-gated cation channel that enhances Ca^{2+} permeability (Blanke and Van Dongen 2009). The NMDAR is critical in the

development of the central nervous system (CNS). Their hypofunction can lead to cognitive decline and over functioning leads to the degeneration of neural cells (Blanke and Van Dongen 2009). The NMDAR is a high voltage-dependent receptor and gets activated on binding with glutamate, causing an influx of Ca^{2+} into the cytoplasm. However, the NMDARs play a complex and critical regulatory role and normally prevent the entry of Ca^{2+}. Intracellular Ca^{2+} is responsible for cell death. Another important receptor, the sigma receptor ($\sigma - 1$), prevents the uptake of Ca^{2+} by the NMDA receptor from the extracellular space. Their agonists also sustain the mitochondrial membrane potential and prevent the release of the cytochrome C oxidase, the key protein in the oxidative phosphorylation preventing damage from the reactive oxygen species (ROS). They also work with voltage-dependent Ca^{2+} channels and prevent the uptake of Ca^{2+}. Sigma receptors co-localize with the inositol triphosphate (IP_3 receptors) and prevent stress on the mitochondrial endoplasmic reticulum. Because of their affinity for a wide spectrum of ligands, they are believed to be amplifiers of intracellular transduction (Blanke and Van Dongen 2009). Tumour necrosis factor-alpha (TNF-α) through its receptors on the cell membrane activates cell apoptosis resulting in the cell death. Endothelin (ET-1) enhances glutamate-induced retinal nerve cell death through ET_A receptors especially under hypoxic conditions (Kobayashi et al. 2005).

Neurotrophins (NT) on the other hand are neuroprotective agents that are critical for cell survival in the CNS and the peripheral nervous system. The NTs work by activating the tyrosine kinase (Trk) receptors on the cell surface, which act as a ligand for the NTs and lead to MAP kinase cascade, the signalling pathways for cell survival. Without the support of NTs, the neurons undergo apoptosis and degenerate. NTs are expressed both in the brain and the retina. In glaucoma, there is a reduced expression of NTs in the retina (He et al. 2018).

5.2.2.2 Translation of Neurotrophins Research in Glaucoma

The Ciliary Neurotrophic Factor

The ciliary neutrophic factor (CNTF) belongs to the IL-6 family of cytokines and binds to CTNFR. In a preclinical rat model of glaucoma, CNTF reduced the loss of the RGC by activation of JAK-STAT3 pathway (Ji et al. 2004). Similar preclinical rat model studies have shown the efficacy of various other neurotrophic factors such as brain-derived neurotrophic factor (BDNF), the nerve growth factor (NGF) and glial-derived neurotrophic factors in preventing RGC loss or promoting survival of these cells (Guymer et al. 2019). Using cell encapsulation technique, the CNTF gene-transfected cells were successfully implanted in human eyes (Sieving et al. 2006). Neurotech Pharmaceuticals has developed a device, NT-501(Renexus®), based on cell encapsulation technique in which RPE cells transfected with CNTF gene are sequestrated in a semipermeable hollow fibre and when implanted in the eye release CNTF. A phase 2 sham-controlled clinical trial that enrolled 54 patients of glaucoma is underway [https://www.prnewswire.com/news-releases/neurotech-pharmaceuticals-inc-and-lowy-medical-research-institute-announce-publication-of-nt-501-phase-2-results-300778781.html accessed 15 Oct 2020].

The Nerve Growth Factor

The nerve growth factor (NGF) is one of the earliest and a well-known neurotrophic factor discovered more than 50 years ago. A phase Ib study to evaluate the safety and potential efficacy of a topical recombinant human nerve growth factor (rhNGF) as rhNGF eye drops (180 μg/mL) has been completed in 2019 in patients with glaucoma (NCT02855450) at the Byers Eye Institute at Stanford University and the results are awaited.

The Brain-Derived Neurotrophic Factor

The brain-derived neurotrophic factor (BDNF) produced in the lateral geniculate nucleus reaches the RGC in a retrograde flow along the axons of RGC. The BDNF rescues neurons from a variety of insults including ischaemic, traumatic or toxic brain injury. Moreover, higher levels of BDNF proteins resist damage by ischaemia. BDNF acts by its affinity for TrkB receptors and protects neurons from glutamate-induced apoptosis (Almeida et al. 2005). In a spontaneous mouse model of glaucoma, intravitreal injection and more so the topical BDNF were found to be a safe and effective strategy in preventing RGC loss and led to the recovery of the pattern ERG as well as the VEP (Domenici et al. 2014). In a previous study, transfection of RGC in a rat model with the vector adeno associated virus (AAV) to express BDNF was found to protect the RGC from IOP elevation and found to have a potential role as a complementary therapy in human glaucoma (Martin et al. 2003).

The major challenge of BDNF therapy either through a recombinant protein or through an AAV vector is the lack of sustenance of effect because over time the BDNF receptor, the TrkB, shows downregulation. Osborne et al. (2018) described a novel approach by using AAV2 TrkB-2A-mBDNF that increased the BDNF production as well as increased the expression of TrkB in the inner retina. The treatment was effective for over 6 months in a mouse model of glaucoma and the optic nerve injury and did not show any adverse effects on the functioning of retina paving the way for human trials (Osborne et al. 2018). However, to date, no registered clinical trial using this gene therapy has been initiated. Interestingly, the use of conventional IOP lowering topical therapies with prostaglandin analogues, carbonic anhydrase inhibitors or β-blockers itself may lead to activation of the neuroprotective pathways as was seen in an immunohistochemical study of eight donor retinas from glaucoma patients, and nine normal retinas that topical drugs used to treat glaucoma were associated with an increase in the retinal BDNF and TrkB expression in the human retina, and there was no difference in glaucoma and normal retinas (Harper et al. 2020).

5.2.2.3 Translation of Neurotrophins Research in Macular Telangiectasia

Macular telangiectasia type 2 is a progressive degenerative disorder of the macula resulting from degeneration of Müller cells for which no genetic factor has been identified so far (Powner et al. 2010). The CNTF, a non-specific neuroprotective agent, was shown to act by activation of receptor gp130 on Müller cells, and its

protective role was demonstrated in preclinical models of retinal degeneration (Duncan 2019; Rhee et al. 2013).

In phase II multicentric sham-controlled trial that enrolled 99 eyes of 67 patients with macular telangiectasia type 2, a sustained release device (Neurotech-501/ Renexus® implant) that produced 20 ng/day of CNTF in the vitreous cavity slowed down the progression of the disease compared to the sham group over 24 months of follow-up (Chew et al. 2019). Serious adverse events included persistent delayed dark adaptation and miosis in 19% of the eyes and were most likely related to the CNTF. Currently, a sham-controlled, multicentric phase III trial (NCT03319849) is underway in the USA to see the efficacy and safety of Renexus® implant. The study is likely to be completed by August 2022.

5.2.2.4 Translation of Neurotrophin Research in Dry Eyes and Other Diseases

Very recently topical drops of rhNGF have been found safe and effective in moderate to severe neurotrophic keratitis (Bonini et al. 2018) and found safe in the dry eyes as well (Sacchetti et al. 2020). Potential role of rhNGF is being studied in retinitis pigmentosa (NCT02110225) and even cataract and refractive surgery (NCT03035864).

5.3 Growth Factors and Their Inhibition in Ocular Disorders

5.3.1 The Discovery of Vascular Endothelial Growth Factor

The year 1989 is the watershed year when the path-breaking discovery of vascular endothelial growth factor (VEGF) led to a revolutionary shift in the treatment paradigms in eye diseases and unfolded the story of the bench-to-bedside research, the true translational research in ophthalmology (Ferrara and Henzel 1989; Connolly et al. 1989; Leung et al. 1989; Ferrara 2016). In 2010, Napoleone Ferrara received the Lasker-DeBakey Clinical Medical Research Award for his work on the discovery of the VEGF. The VEGF is a fundamental gene in embryonic physiology for normal vasculogenesis, haematopoiesis and organogenesis. It also gets upregulated whenever there is tissue hypoxia or increased oxygen demand and leads to pathological angiogenesis as seen in tumours and intraocular neovascularization as seen in neovascular age-related macular degeneration (nAMD) and ischaemic retinal diseases such as diabetic retinopathy (DR) and retinal vascular occlusions (RVO). The VEGF was discovered in 1996 as the main driver of nAMD as well (Lopez et al. 1996; Kvanta et al. 1996; Frank et al. 1996). The VEGF leads to vascular endothelial growth, increased permeability and recruitment of leucocytes, all contributing to a pathology consistent with nAMD (Ferrara 2004).

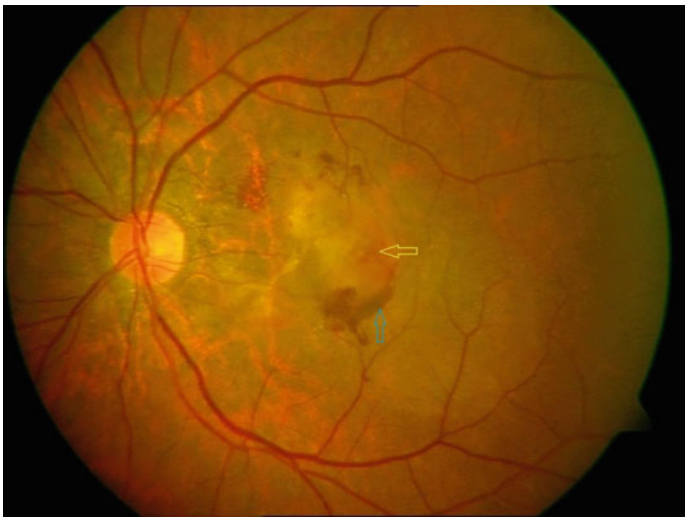


Fig. 5.6 Retinal image of the left eye of a patient with wet type of age-related macular degeneration (nAMD). Blue arrow shows extensive haemorrhage under the neurosensory retina. The yellow arrow points to the accumulation of turbid fluid under the retina. Macula has lost transparency. These patients have a sudden-onset vision loss

5.3.2 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a common vision limiting and disabling disease of the old age with a global prevalence of nearly 190 million and is a leading cause of blindness in the western world. Basically it is of two types: (1) the dry type which accounts for nearly 90% of all AMD patients which is a slow bilateral progressive disease with degeneration of the RPE and the overlying photoreceptors in the macular area, eventually causing loss of central vision with an attendant inability to read, recognize and see colours by the patient, and (2) the wet type, which in nearly 10% of patients, abnormal blood vessels from the choriocapillaris grow under the RPE or the neurosensory retina or both, and leak fluid, blood and exudates and depending upon the location of these vessels in the macula can cause an abrupt onset of moderate to severe visual loss (Fig. 5.6). This is commonly called neovascular age-related macular degeneration (nAMD).

Molecular mechanisms of nAMD: Apte, Chen and Herrara recently reviewed the underlying molecular mechanisms of nAMD (Apte et al. 2019). Briefly, under hypoxic conditions, the VEGF is expressed by microglia and Muller cells (the macroglia) in the retina which leads to the development of abnormal vessels under or even in the neurosensory retina. These vessels do not have tight junctions as in the normal retinal vasculature and leak fluid and blood and, if allowed to stay for a while, invite scar formation. Besides the VEGF-165 isoform, there are other growth factors like placental growth factor (PlGF), platelet-derived growth factor (PDGF) or angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) which play a role in nAMD and

can be effectively blocked by antibodies but only for a limited duration, hence the need for alternate strategies (Guimaraes et al. 2021).

5.3.3 Development and Application of the First Anti-VEGF Antibody (Avastin)

The discovery of VEGF was followed by the development of its humanized antibody bevacizumab (Avastin) by Ferrara in 1997 while he was working with Genentech. This seminal discovery led to a revolutionary change in the treatment paradigm of retinal diseases, namely, the nAMD, the diabetic retinopathy and retinal vein occlusions (Ferrara 2011). In Feb 2004, use of Avastin was US FDA approved as an adjunct therapy for patients with metastatic colorectal carcinoma. (Michels et al. 2005). It took almost a decade for Philip Rosenfeld and his colleagues to use Avastin in patients with nAMD. In an uncontrolled study, nine patients were administered an intravenous infusion of the anti-VEGF molecule bevacizumab (Avastin; Genentech) (5 mg/Kg body weight). The treatment was successful and led to an improvement in vision, structural OCT changes and fluorescein angiographic changes. Systemic therapy was well tolerated (Michels et al. 2005). Before the application of the anti-VEGF therapy in humans, Genentech had developed another molecule ranibizumab (Lucentis; Genentech), an anti-VEGF antibody fragment specifically designed for intraocular use. It had been shown in 2002 to prevent the development of choroidal neovascularization in a monkey model providing the first preclinical proof of concept for use of anti-VEGF therapy in nAMD (Krzystolik et al. 2002). How the first uncontrolled use of bevacizumab came to be done in humans for nAMD, even as the phase I/II controlled trials for another molecule Lucentis (Genentech) were underway and the results would not be known for another 2 years, has been told by Dr Rosenfeld [https://retinatoday.com/articles/2009-may-june/0609_05-php].

Phase I/II trial of another anti-VEGF aptamer, pegaptanib (Macugen), was also reported in 2002 (Eyetech Study Group 2002, 2003).

At the same time, two major phase III human trials, the Anchor and the Marina that would go on for 2 years, were initiated to test the efficacy of monthly intravitreal (IVT) injections of ranibizumab in nAMD. At the 18 months follow-up, the improvement in visual acuity that was seen initially had been maintained. These two pivotal trials would later go on to show that monthly injections for nAMD led to improvement or stabilization of visual acuity in 95% of the patients and become the standard of care (Michels and Rosenfeld 2004).

5.3.4 VEGF-Trap (Aflibercept, Regeneron)

With the discovery of soluble growth factor, VEGF, initial attempts had been made to block the target VEGF receptors on the endothelial cells using soluble monoclonal antibodies. Holash et al. (2002) reported preparing a fusion protein that had a remarkable affinity for the VEGF receptors and effectively suppressed

vascularization and tumour growth. Moreover, it also extended its in vivo half-life. The VEGF-Trap was created by fusing the first three Ig domains of VEGFR1 and VEGFR2 to the constant region (Fc) of human immunoglobulin, IgG1 (Holash et al. 2002). VEGF-Trap binds tightly to VEGFR-A, and unlike Lucentis or Avastin, it blocks all isoforms of VEGF-A and VEGF-B and additionally binds the placental growth factor (PlGF). It prevents the VEGF to activate its receptors on the endothelial cells. It is also known to reverse leucostasis in DR. In two parallel double-masked, active-controlled (Lucentis) randomized trials in nAMD, VIEW1 and VIEW2, IVT aflibercept injections given 2-monthly after the initial three once-monthly injections were as effective and safe as Lucentis given as monthly injections (Heier et al. 2012). Moreover, aflibercept had the added advantage of reducing the potential risk associated with monthly injections.

5.3.5 Placenta Growth Factor (PlGF)

Angiogenesis is a complex process during the development of the embryo; however, in the postnatal period, it plays a crucial role only during menstruation and in the heart and the skeletal muscles during strenuous exercise. Placental growth factor (PlGF) was the second discovered member of the VEGF family in 1991. While in physiological conditions its role is almost redundant but under pathological conditions such as cancers, diabetic retinopathy and the age-related macular degeneration, it plays a key role in the development of abnormal vessels (De Falco 2012). An anti-PlGF recombinant humanized monoclonal antibody, the THR-317 against the receptor-binding site of the human placental growth factor, has been developed by Oxurion NV. The phase1/2 top-line data presented in 2019 showed it to be a safe and effective drug for anti-VEGF-naive diabetic macular oedema [https://www.oxurion.com/sites/default/files/upload/news/OXUR%20PR_FLORetina2019%20THR317%20Ph12_EN.pdf]. A multicentric phase II study (NCT03669393), sponsored by Thrombogenics/Oxurion to assess the safety and efficacy of THR-317, an anti-PlGF recombinant humanized monoclonal antibody delivered through three intravitreal injections (8 mg) 1 month apart in macular telangiectasia type1, was completed in Nov 2019 and the results are awaited.

5.3.6 Anti-VEGF Therapies: The Standard of Care for nAMD

Subsequent to these pioneering studies, intraocular injections of anti-VEGF molecules such as bevacizumab, ranibizumab and aflibercept have become the standard of care in ophthalmology. These agents lead to a dramatic regression of the abnormal vessels in nAMD and have saved eyesight of millions of patients all over the world. This therapy is currently the gold standard of treatment in nAMD. However, as noted even in the initial clinical studies, the effect of these molecules lasts only for about 4 weeks and the injection needs to be repeated every month all through life. Although the anti-VEGF injections restore the lost vision to a great

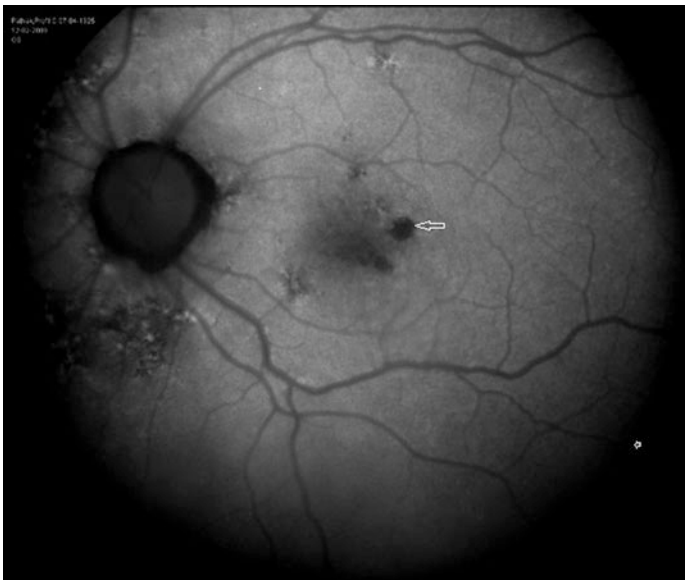


Fig. 5.7 A fundus autofluorescence image of the left eye in an 85-year-old patient with nAMD shows an area of reduced fluorescence due to loss of RPE and the overlying photoreceptors (arrow). This is called geographic atrophy (GA). The abnormal new vessels under the fovea responded favourably following intraocular injection of anti-VEGF drug Avastin given every month (image not shown)

extent but at the cost of the increased burden on the caregivers and the patients by increasing the number of hospital visits. A systematic review of the real-world current practices in the UK of managing nAMD shows that the number of injections (data only for Lucentis and Eylea) in the first year varied from 5.9 in 2010–2015 to 7.1 in 2015–2020 (Mehta et al. 2020). Moreover, long-term follow-up has revealed that initial improvement of vision seen in the first year of anti-VEGF therapy continues to deteriorate later on due to progression of the degenerative changes in the macular area (Figs. 5.7 and 5.8).

5.3.7 Diabetic Retinopathy (DR)

Diabetes mellitus (DM) is a major public health challenge affecting nearly 4.7–8.5% of the population especially in the lower- and middle-income countries. In 1980, 108 million people were living with DM, and by 2014, there were 422 million such affected people. DM is not only the leading cause of death but a leading cause of blindness as well in people in the working-age group [https://www.who.int/news-room/fact-sheets/detail/diabetes].

Long-standing out-of-range blood sugar levels lead to target organ damage including the retina, kidneys, peripheral nerves, autonomic nerves system and

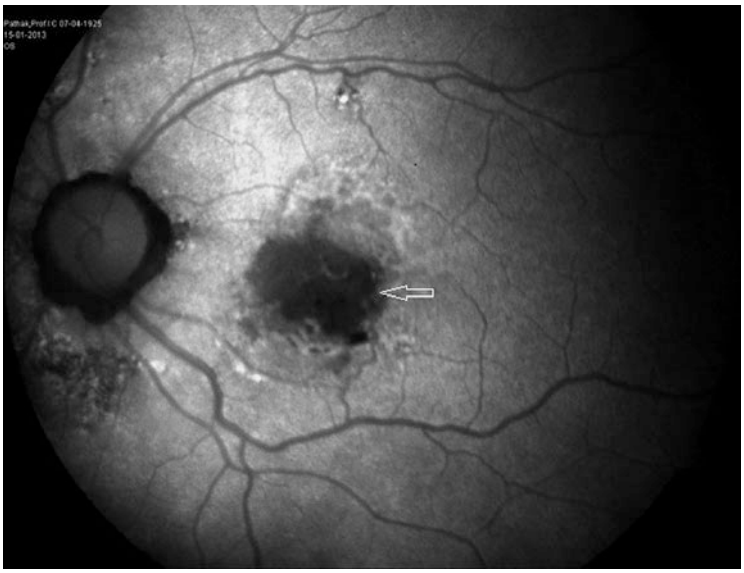


Fig. 5.8 Fundus autofluorescence image of the same eye as in Fig. 5.7 obtained 4 years after the patient had received nearly 48 injections of Avastin given every month. Note the progression of GA (arrow) despite control of the abnormal vessels

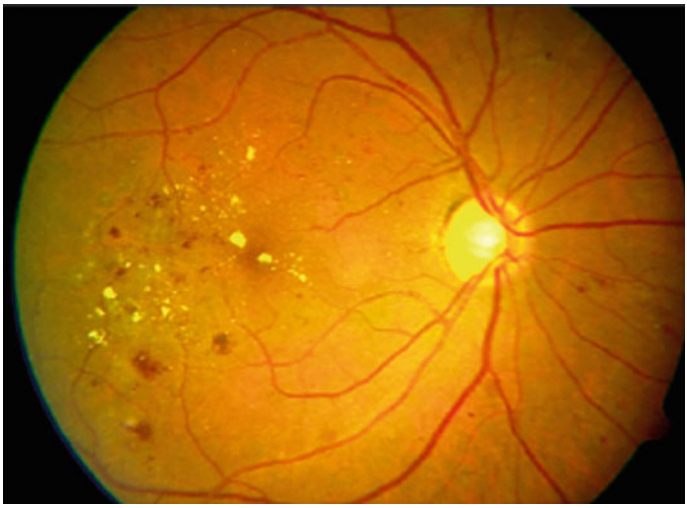


Fig. 5.9 Retina in a patient with diabetes mellitus (DM) shows microaneurysms (red dots), small haemorrhages and lipid deposits in the macula of the right eye. This is diabetic macular oedema and is the commonest cause of moderate visual impairment in patients with DM

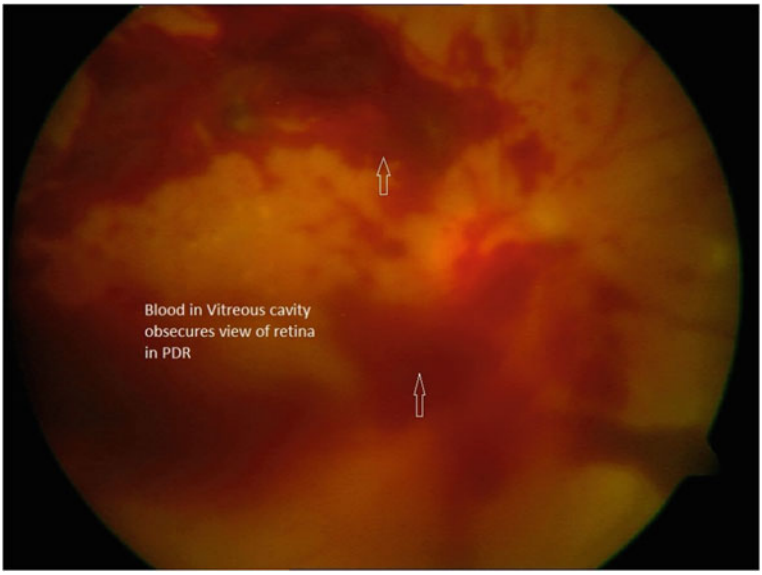


Fig. 5.10 There is blood in the vitreous cavity due to bursting of the abnormal new vessels on the optic nerve head or elsewhere in the retina causing sudden moderate to severe loss of vision

cardiovascular system. Nearly one-third of people with DM have their retinas affected at any given point of time (prevalence). DM leads to the formation of retinal microaneurysms, loss of the inner blood-retinal barrier and retinal capillary closure with consequent leakage of fluid, RBCs and lipoprotein exudates in the extracellular space resulting in diabetic retinopathy (Fig. 5.9). Collection of fluid in the macula is called macular oedema and is the commonest cause of visual impairment in patients with DM. Extensive retinal capillary closure over time leads to the formation of abnormal new vessels resulting in proliferative diabetic retinopathy (PDR). Bursting of abnormal retinal vessels leads to haemorrhage in the vitreous cavity causing sudden severe impairment of vision (Fig. 5.10). Abnormal vessels are accompanied by scar tissue which contracts and leads to traction on the retina resulting in retinal detachment. Untreated DR results in blindness.

The status of the retinal perfusion and the leaking microaneurysms is determined by fundus fluorescein angiography (Fig. 5.11). For more than 50 years, the standard of care has revolved around destroying the leaking microaneurysms and non-perfused ischaemic retina with laser photocoagulation to prevent blindness (Fig. 5.12).

5.3.8 Pioneering Studies on the Role of VEGF in DR

Michaelson, in 1948, first proposed a diffusible ‘X’ factor from the ischaemic retina that led to pathological angiogenesis in the eye of patients with DM (Patz 1984).

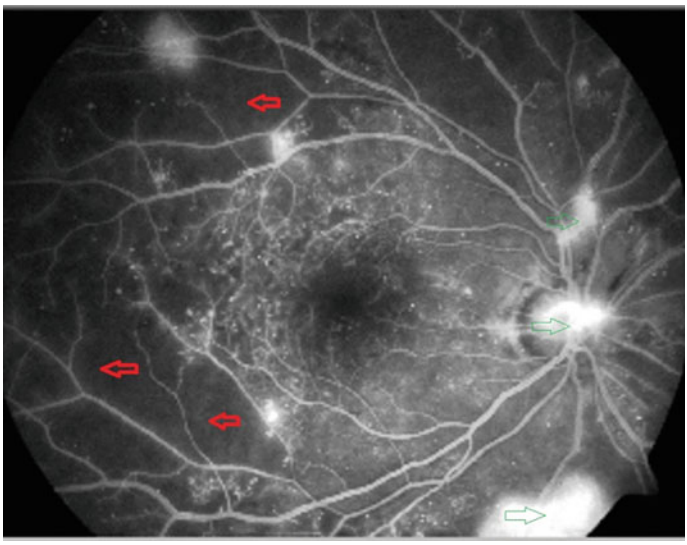


Fig. 5.11 Fundus fluorescein angiography image in a patient with DM obtained after intravenous injection of sodium fluorescein dye. There are areas of retina that do not have capillaries (red arrows) causing retinal ischaemia and is responsible for the release of VEGF which drives the formation of multiple areas of abnormal retinal vessels (green arrows) called proliferative diabetic retinopathy (PDR)

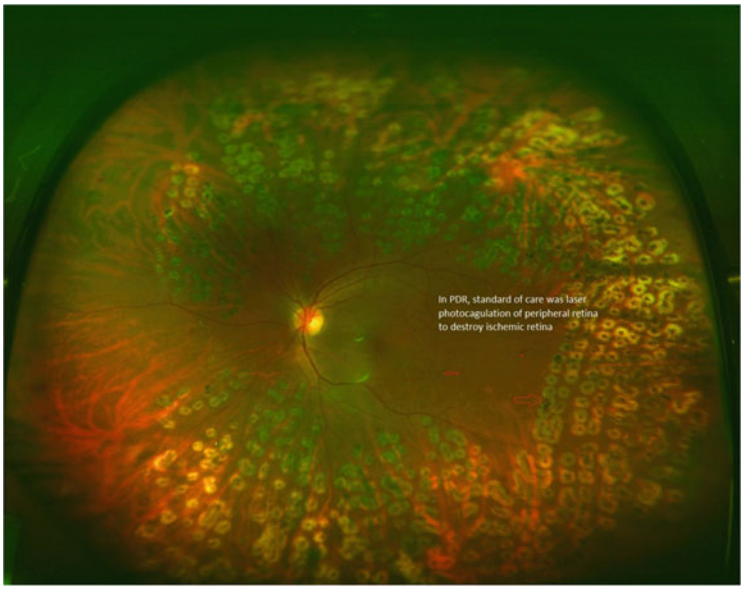


Fig. 5.12 Retinal image of a patient with DM who has undergone laser photocoagulation of the retinal periphery to ablate the ischaemic retina to prevent the release of VEGF from the ischaemic retina. This is a destructive procedure and causes restriction of the peripheral field of vision. Till recently, it was the standard of care for PDR

Following the discovery of this diffusible 'X' factor as the VEGF by Ferrara in 1989, the upregulation of VEGF was found in the ocular fluids in a primate model of retinal ischaemia providing evidence for the existence of Michaelson's hypothesis (Miller et al. 1994). The upregulated soluble VEGF was also detected in the intraocular fluids of patients with diabetic retinopathy (Aiello et al. 1994). Tolentino et al. (2002) succeeded in producing many of the clinical features of non-proliferative (NPDR) and proliferative DR (PDR) in a non-human primate model by injecting VEGF into the vitreous cavity, thus paving the way for testing novel therapies in DR. One of the earliest molecule to be tested in diabetic macular oedema (DME) was IVT injection of a synthetic pegylated (to increase its stay in the vitreous cavity) aptamer of VEGF-165, pegaptanib (Macugen). Preliminary data suggested that Macugen was effective in reducing the macular thickness and led to an improvement of vision (Cunningham Jr et al. 2005). These results were confirmed by a phase II/III study (Sultan et al. 2011). Macugen was also found to be effective in delaying or preventing vitreous surgery for non-clearing vitreous haemorrhage (Hornan et al. 2010). However, there were concerns with the use of anti-VEGF agents in patients with DM, as these patients are, in any case, more susceptible to cardiovascular and cerebrovascular events. Pooled study of all pegaptanib nAMD trials, although the study population was skewed as only 10% of the nAMD patients enrolled in these trials were living with DM, revealed somewhat higher vascular events based on the Antiplatelet Trialists' Collaboration (APTC) criteria at 6.1% in DM versus 4.2% in non-DM nAMD patients (Dombi et al. 2012).

Earlier, systemic administration of Avastin in high doses that are typically used in oncology patients had also resulted in a high incidence of hypertension, GI perforations, thrombosis and haemorrhages (Kamba and McDonald 2007). In July 2011, Pfizer withdrew the drug for its application for DME due to its failure to show a positive benefit-risk balance [https://www.ema.europa.eu/en/documents/other/macugen-withdrawal-letter_en.pdf].

In 2007, fractionated from a 40 mg/mL vial and hence a much cheaper alternative for the patient, 1.25–2.5 mg/0.1 mL IVT Avastin injection was shown to stabilize or improve the visual acuity, OCT and FFA changes for at least 6 months in patients with DME (Arevalo et al. 2007). Millions of patients with DM world over even now continue to receive IVT injections of Avastin (2.5 mg/0.1 mL) because of its almost similar efficacy and huge cost differentials from the other anti-VEGF therapies like Lucentis and aflibercept (Eylea).

5.3.9 Seminal Phase III Trials on the Use of Lucentis in DME

Two parallel identical phase III RISE (NCT00473330) and RIDE (NCT00473382) studies in patients with DME showed a rapid and sustained improvement in visual acuity and reduced macular oedema following an IVT injection of 0.3 mg or 0.5 mg/month of Lucentis over 2 years of follow-up. However when injected into the vitreous cavity, the anti-VEGF agents find their way into the systemic circulation, and there is a risk of blocking the physiological functioning of the VEGF. Serious

adverse events from systemic inhibition of VEGF including death, non-fatal cerebrovascular events and non-fatal myocardial infarctions varied from 2.2 to 8.8% in the Lucentis group versus 4.9 to 5.5% in the sham group (Nguyen et al. 2012). The US FDA approved the use of Lucentis for DME on August 10, 2012 [https://visionaware.org/blog/visionaware-blog/the-fda-approves-lucentis-for-diabetic-macular-edema/]. In an open-label extension of the RISE and RIDE trials from 36 to 48 months, 70% patients who received the IVT injection of Lucentis 0.5 mg, on a *pro re nata* basis, showed a consistent improvement in DR severity scores (Sun et al. 2019).

5.3.10 Anti-VEGF Lucentis Therapy for Proliferative Diabetic Retinopathy

Since the early 1970s, the standard-of-care treatment for PDR has remained pan-retinal photocoagulation (PRP) using ophthalmic lasers (Fig. 5.12). However, besides worsening the leakage from the retinal capillaries in the macula and thereby worsening the coexisting macula oedema, laser photocoagulation being a retina destructive procedure leads to restriction of the peripheral field of vision and reduced visibility in the dark. Once the efficacy and safety of the anti-VEGF therapy had been established for DME, a multicentric controlled trial by a group of retina specialists, in the USA, namely, the Diabetic Retinopathy Clinical Research Network (DRCR.net), tested the non-inferiority of Lucentis versus PRP and found that through the 2-year follow-up, Lucentis was non-inferior to the standard-of-care PRP and in fact led to significant improvement in vision compared to the PRP (Writing Committee for the Diabetic Retinopathy Clinical Research Network et al. 2015). Lucentis was found to be a reasonable and safe alternative to the destructive laser therapy, and on April 17, 2017, the US FDA approved the use of Lucentis for the treatment of PDR (Figs. 5.13 and 5.14) [https://visionaware.org/blog/visionaware-blog/the-fda-approves-lucentis-for-the-treatment-of-diabetic-retinopathy/].

5.3.11 Anti-VEGF Eylea Therapy for Proliferative Diabetic Retinopathy

In two parallel randomized controlled phase III trials, VISTA and VIVID, the use of either 2 mg/4 weeks or 2 mg/8 weeks after five monthly injections of IVT aflibercept IVT injection in patients with DME led to significant improvement in visual acuity (mean +10–11 letters; >40% had >15 letters) compared to the laser photocoagulation (mean +1.4 letters, 13–19%) at 3 years. There was a significant improvement in DR severity score as well (Heier et al. 2016). More recently, 2 years results of PANORAMA trial of IVT Eylea given every 16 weeks showed substantial improvement in DR severity score in patients with moderately severe to severe non-proliferative DR as well (https://www.healio.com/news/ophthalmology/20190329/early-intervention-with-eylea-has-positive-results-in-panorama-trial).

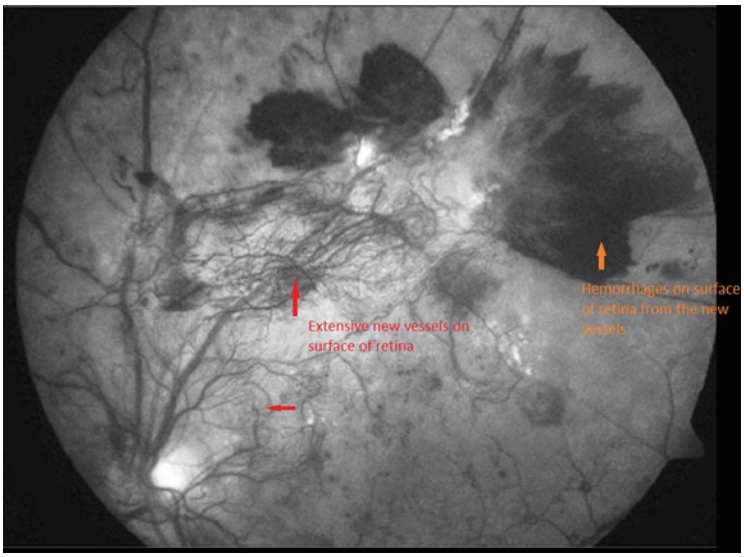


Fig. 5.13 Left eye retina of a patient with PDR. There is extensive network of abnormal vessels which have started bleeding. He was the first patient of the author to receive intravitreal injection of 2.5 mg of Avastin in early 2006

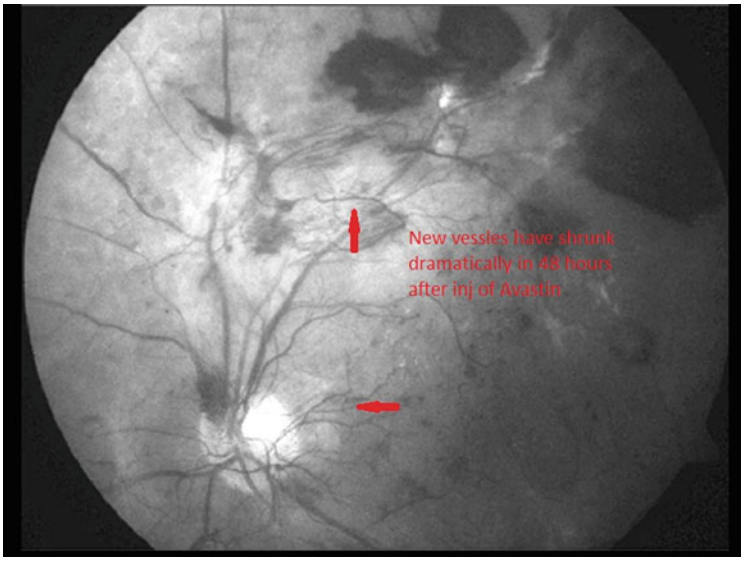


Fig. 5.14 The same eye as shown in Fig. 5.13, after 48 h of Avastin injection into the vitreous cavity, shows a dramatic disappearance of most of the abnormal retinal vessels. The abnormal retinal vessels need a continuous supply of VEGF to sustain them. Anti-VEGF antibodies like Avastin, Lucentis or Eylea block the free VEGF leading to disappearance of the vessels. However the effect of one injection lasts for only 4 weeks or so

The US FDA, on May 13, 2019, approved Eylea for treatment of nAMD, DME, all stages of DR and macular oedema secondary to central retinal vein occlusion (https://investor.regeneron.com/news-releases/news-release-details/fda-approves-eylear-aflibercept-injection-diabeticretinopathy#:~:text=EYLEA%C2%AE%20(aflibercept)%20Injection%202,and%20Diabetic%20Retinopathy%20(DR)).

5.3.12 New Agents in Development: Blocking the Angiopoietin-Tie Signalling Pathway

New agents are being tested that target the Angiopoietin-Tie (tunica interna endothelial kinase) signalling pathway that controls vascular endothelial permeability and stability. Ang-2 is an inhibitor of Tie-2 gene. Blocking Ang-2 activates Tie-2 gene resulting in decreased vascular permeability (Zhang et al. 2019). In phase II, multicentric controlled trial (NCT02699450), monthly IVT injections of a combination of Ang-2 and VEGF-A blocking drug, faricimab, for 20 weeks led to superior visual acuity outcomes (+3.6 letters) and structural outcomes versus the Lucentis at 36 weeks (Sahni et al. 2019).

5.4 Gene Therapy

Extensive research has been done in the last 30 years to unravel the molecular mechanisms and genetics of several blinding diseases of the eye for which either the treatment is ineffective or not available. Recently, there has been an upsurge in translating this information for application in patient care in several preclinical and clinical studies. Most prominent of these have been in inherited retinal disorders (IRD) and age-related macular degeneration (ARMD) affecting the outer retina, glaucoma affecting the retinal ganglion cells (RGC) and their axons in the inner retina and diabetic retinopathy affecting the retinal microvasculature.

5.4.1 Inherited Retinal Degenerations

Inherited retinal degenerations (IRD) affect millions of patients worldwide and nearly half of them are caused by retinitis pigmentosa (RP), a progressive degenerative condition of the retinal photoreceptors which causes night blindness in the beginning eventually leading to irreversible total blindness (Figs. 5.15, 5.16, and 5.17). The invention of ophthalmoscope by Hermann von Helmholtz (1851) led to the discovery of RP and the clinical features of the disease were described soon after. Although it is a non-inflammatory disease, the name retinitis pigmentosa first suggested by Donders (1855) has stuck (Duke-Elder and Dobree 1967). While Gregor Mendel and Charles Darwin were laying down the basic principles of the biology of heredity in the 1860s, von Graefe (1858) and Liebreich (1861) had already observed the occurrence of RP in families (Duke-Elder and Dobree 1967).

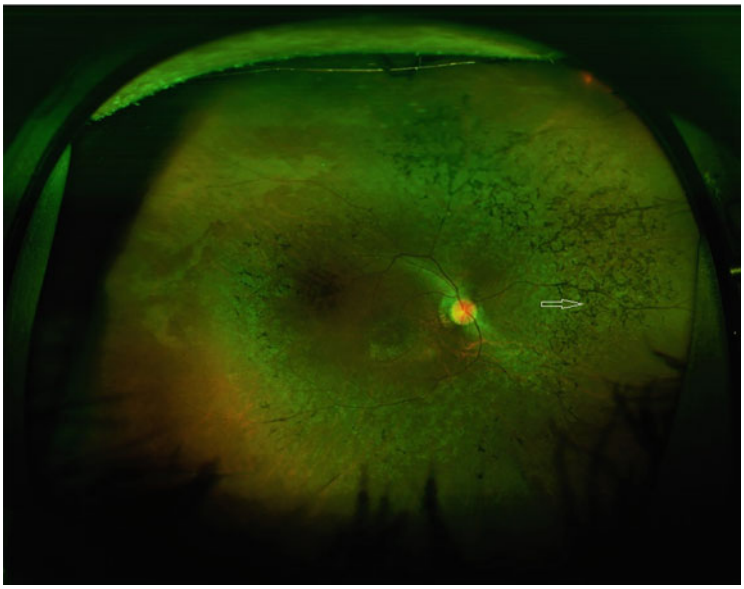


Fig. 5.15 A 30-year-old male patient suffered from progressive blindness in both eyes since early childhood. His elder brother had a similar disease. He was diagnosed as retinitis pigmentosa. The retinal image of the right eye shows bone-spicule pigmentation, waxy pallor of the optic nerve head and attenuation of the retinal vessels (Image courtesy: Dr Reema Bansal)

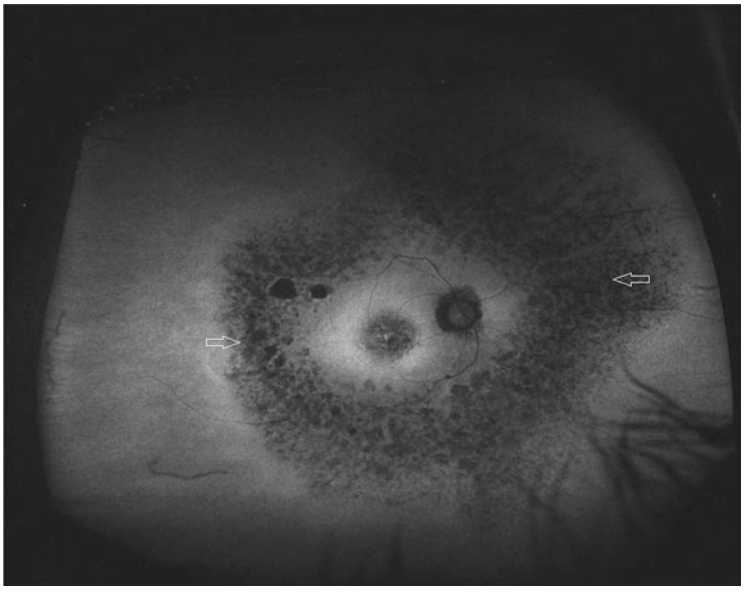


Fig. 5.16 Fundus autofluorescence image of the same eye as shown in Fig. 5.15. Extensive dark areas indicate loss of the RPE and the overlying photoreceptors (Image courtesy: Dr Reema Bansal)

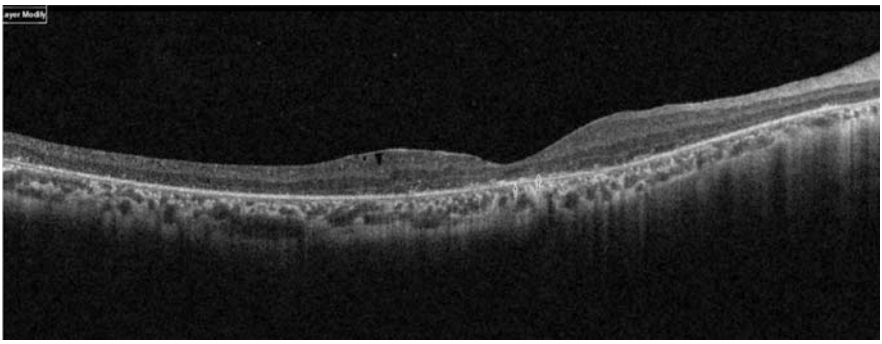


Fig. 5.17 The OCT image of the same eye as shown in Fig. 5.15 shows the disappearance of the RPE and photoreceptors. Note the contrast from the line scan from a normal person shown in Fig. 5.3 (Image courtesy: Dr Reema Bansal)

Nettleship (1907–1908), in a large cohort of families, did not find evidence of heredity in nearly half of the patients but a quarter each had either consanguineous or non-consanguineous parents (Duke-Elder and Dobree 1967). For the next 100 years, the focus remained on the clinical phenotypes and patterns of Mendelian inheritance. The autosomal recessive pattern emerged as the most common variant with an earlier onset and more severe disease than the autosomal dominant which was less common and the X-linked as rare (Duke-Elder and Dobree 1967). The late 1980s saw the creation of databases of all patients with IRD especially in Philadelphia (all ages, mostly adults) and Toronto (children <16 years). This database would be utilized for genotyping. Till recently there has been no known treatment for this disorder. Discovery of the rhodopsin gene in the 1970s was followed by extensive genetic studies to discover more than 300 genes and loci for the IRD with thousands of mutations [https://sph.uth.edu/retnet/sum-dis.htm accessed 20.10.2020].

Retinitis pigmentosa can be syndromic or non-syndromic and is one of the most heterogeneous diseases. It may have different disease manifestations in the same family. Different genes may cause the same clinical disease or the same mutations in the gene may cause a different disease and even different mutations in the same gene may cause a different disease expression (Daiger et al. 2013). Leber congenital amaurosis (LCA2) is a rare autosomal recessive form of progressive RP which leads to blindness in early years of life. It is caused by biallelic variation in a single gene RPE65 that codes for a protein expressed in the RPE cells. This enzyme isomerizes all-trans-retinyl esters to11-cis retinal, a step in the visual pigment regeneration in the photoreceptors, a critical step in the visual cycle (Fig. 5.18) [https://www.ncbi.nlm.nih.gov/gene/6121 accessed 20.10.2020].

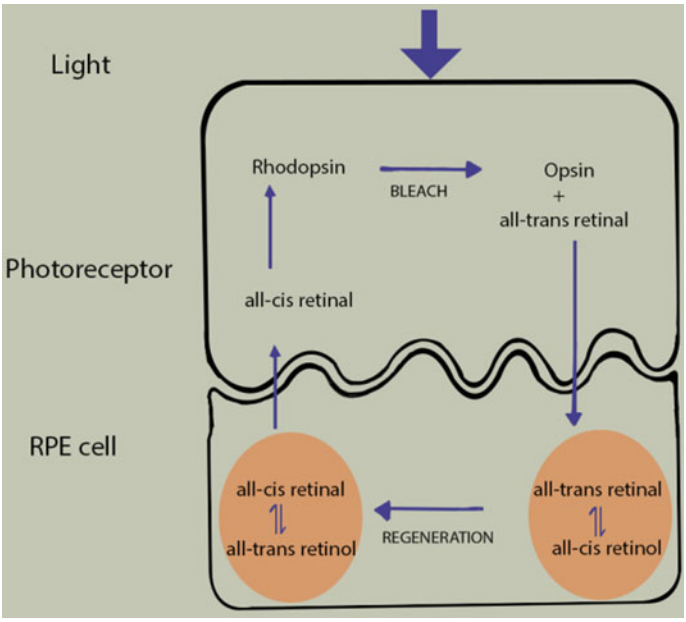


Fig. 5.18 Highly schematic sketch of the visual cycle to show the bleaching of the photopigment (rhodopsin) in the photoreceptor cell and its regeneration in the RPE cells (Graphics: Kritika Thakur)

5.4.2 First Gene Therapy Approved for LCA

More than two decades of exhaustive bench research finally led to a landmark approval by the US FDA in Dec 2017 for application of the first adeno-associated virus (AAV)-based gene supplement therapy in patients with LCA using Luxturna (voretigeneneparvovec-rzyl, Spark Therapeutics), an RPE 65 gene. Alexandra Garafalo et al. (2020) recently reviewed the journey of the last three decades in determining the Mendelian genetics in patient cohorts of IRD and molecular genetics and estimating the frequency of the genetic mutations that are responsible for the IRDs, the most prominent of which is RP. Nearly half of the cohorts still have unresolved mutations due to rarity of their occurrence. They also summed up how the bench work was translated from preclinical to studying the safety of the vectors and finally reaching the stage of patient trials (Garafalo et al. 2020). Following a single subretinal injection of Luxturna, the treatment was found safe and led to the maximal improvement in visual parameters at day 30 that was maintained up to 4 years of the injection. No adverse immunological reactions were encountered (Maguire et al. 2019).

The success of this therapy has led to a wave of optimism among millions of patients who are blind from IRDs even prompting a change in the terminology to provide for more specific information to all stakeholders (Pennesi and Schlecther 2020). Gene augmentation or gene replacement is preferred over the non-specific

'gene therapy' to convey the intention of the therapy. Also, the clinical diagnoses are being changed to be more specific genetic defect as exemplified by LCA2 redefined as RPE65 retinopathy. The treatment is, as of now, hugely expensive at $425,000 per eye in the USA (Pennesi and Schlecther 2020). Presently, patients who received Voretigene therapy have been entered into a post-authorization multicentric registry to observe for at least 5 years for long-term safety profile (ClinicalTrials.gov Identifier: NCT03597399).

5.4.3 Clustered Regularly Interspersed Short Palindrome Repeats (CRISPR) Technology

The CRISPR technology was developed by Jennifer Doudna and Emmanuelle Charpentier, the 2020 Nobel laureates, in 2012 (Jinek et al. 2012). It uses a site-specific cleavage enzyme Cas9 to cut through the mutated genes using a guide RNA strand and replace them with healthy genes. Editas Medicine and Allergan have initiated a clinical trial using a subretinal injection of AGN-151587 (EDIT-101), a CRISPR-based strategy to treat LCA10, in 18 patients by targeting a specific gene CEP290. Mutations of this gene are responsible for nearly 20–30% of LCA. The first patient of LCA10 congenital blindness has already been treated using this technology. Previously, using a lentiviral vector, CEP290 replacement was shown in the lab to rescue defective ciliogenesis in precursor photoreceptors from the inducible pluripotent stem cells derived from the fibroblasts of patients who had CEP290-associated blindness (Burnight et al. 2014). However, the adenovirus and the lentivirus vectors both have limitations of transducing in the mature retina (Carrella et al. 2020). One of the major concerns of using CRISPR-Cas9 technology is its unintended off-site targeting of dsDNA (Cho et al. 2018).

5.4.4 Gene Therapy Trials in RP

Initial success of some of the gene therapy studies in patients with RP has led to a flurry of clinical trials. Currently, at least 13 clinical trials have been registered (https://clinicaltrials.gov/ct2/results?cond=Retinitis+Pigmentosa & term=Gene+therapy+OR+CRISPER & type=Intr & rslt= & age_v= & gndr= & intr= & titles= & outc= & spons= & lead= & id= & cntry= & state= & city= & dist= & locn= & rsub= & strd_s= & strd_e= & prcd_s= & prcd_e= & sfpd_s= & sfpd_e= & rfpd_s= & rfpd_e= & lupd_s= & lupd_e= & sort=). The genes being tested include a single subretinal injection of AAV8 vector encoding RPGR (GTPase regulator) proposing to recruit 63 patients of X-linked RP (XLRP), and the study is likely to be completed by Mar 2021 (ClinicalTrials.gov Identifier: NCT03116113).

A study has completed recruitment for a trial of subretinal treatment with AAV2/5-RPGR of XLRP in both children and adults (ClinicalTrials.gov Identifier: NCT03252847). The first-in-human proof-of-concept study is assessing the safety

and efficacy of subretinal injection of CPK850 in patients with RLBP1 (retinaldehyde-binding protein-1) RP due to biallelic mutations in the RLBP1 gene (ClinicalTrials.gov Identifier: NCT03374657). The study is likely to be completed by 2026. While some of the earliest studies required a pars plana vitreous surgery to create access for subretinal delivery of the drug with its attendant surgical complications, newer studies are using a simpler and safer intravitreal route for gene delivery. A proposal includes a trial of gene therapy in 37 patients with intravitreal injection (IVT) of 4D-125 in males with XLRP caused by mutations in the RPGR gene (ClinicalTrials.gov Identifier: NCT04517149).

A first-in-human study to assess the safety and tolerability of IVT injection of QR-421a in patients with Usher syndrome who besides RP also suffer from deafness because of a mutation in Exon 13 of the USH2A gene (ClinicalTrials.gov Identifier: NCT03780257). While biallelic mutations can be targeted with gene supplement strategies, it has been a challenge to target dominant genes. A trial is underway in patients with autosomal dominant RP with a mutant P23H mRNA who shall be given an antisense oligonucleotide (QR-1123) through the IVT route which shall target the mutant mRNA and selectively reduce the expression of the P23H protein while saving the wild-type rhodopsin (RHO) protein. Reduction of the dominant-negative protein is expected to increase the function of the wild-type rhodopsin protein in photoreceptors hoping for the restoration of vision (ClinicalTrials.gov Identifier: NCT04123626).

5.4.5 Gene in Age-Related Macular Degeneration

Many risk factors for AMD have been known for several decades including age above 50 years, race (Caucasians), history of smoking, hypertension, hypermetropic refractive error, a diet lacking green vegetables and fruits and most importantly the family history. The treatment till the first decade of the twenty-first century revolved around the destruction of the abnormal vessels using a variety of lasers that led to a permanent loss of central vision retaining only the ambulatory vision. Drusen, a by-product of low-grade inflammation, and the extracellular deposits between the basement membrane of the RPE and the Bruch's membrane are the hallmark of AMD. Recognition of many components/molecules of the alternate complement pathway in drusen confirmed the role of low-grade inflammation in AMD (Anderson et al. 2010). In a breakthrough in further understanding of the AMD pathogenesis, a defective gene was discovered in patients of AMD, namely, the complement factor H (CFH), a protein that normally controls the immune response and inflammation (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). An SNP in the gene coding for this protein may lead to the diminished blocking effect of this protein. Patients who possessed this defective gene and yet did not develop the disease were found to lack factor B, another important component of the alternate pathway of complement activation (Gold et al. 2006). The two mutations CFH Y402H and LOC387715 A69S along with risk factors like smoking and obesity increase the risk of progressive AMD by 19X (Seddon et al. 2007). In addition to the mutations in the

complement genes described above, an SNP, rs10490924, in the ARMS2 gene located on chromosome 10 is highly associated with ARMD. The functionality of this gene was also shown to be involved in complement pathways mediating the opsonization of necrotic and apoptotic cells (Fritsche et al. 2008; Micklisch et al. 2017). In a two-level model hypothesis, it was recently suggested that the primary insult in the AMD is due to local oxidative stress in the outer retina modulated by genetic mutations which are followed by an inflammatory response to this insult. Hence the strategies to control AMD are designed to target the pathways involved (Rozing et al. 2020).

Age-related eye disease study (AREDS) and AREDS-2 have looked at the role of antioxidant vitamins and Zn+Cu supplements and found a 25% risk reduction in progression to the late stages of AMD, the beneficial effects maintained even up to 10 years (Chew 2020). She found a further additional effect of the beneficial alleles of CFH, but as a marker of the progression to severe AMD, the baseline severity of AMD was a better predictor than the genetic typing.

5.4.6 Complement Inhibition in AMD

Pegcetacoplan is an inhibitor of activation of all the three complement pathways including the classic, lectin and the alternate pathway. In a phase 2b study, use of IVT injection of 15 mg pegcetacoplan every month or every other month led to a significant decrease in the progression of geographic atrophy. While every month treatment was more effective than every other month, nearly 20% of the monthly treated eyes developed new-onset exudative AMD at 12 months versus 1.2% of the sham-treated eyes. Safety and efficacy of this therapy need further evaluation in phase 3 studies (Liao et al. 2020). A C5 inhibitor drug avacincaptad pegol (Zimura, Iveric bio) was tested in a phase 2/3 trial among 286 patients of GA and showed 27–28% reduction in progression rates of GA at 12 months compared to the sham group (Jaffe et al. 2021). However, at the November 2020 annual meeting of the American Academy of Ophthalmology (AAO), the results were updated for the 18 months follow-up to show that 8–16% in the treatment group developed nAMD compared to 2% in the sham group which casts a shadow over the treatment.

Earlier, eculizumab, a systemic inhibitor of complement C5, although well tolerated for 6 months was not found effective in preventing the expansion of geographic atrophy (Yehoshua et al. 2014). Likewise, complement factor D inhibitor lampalizumab given IVT (10 mg) also failed to slow down the rate of GA enlargement (Holz et al. 2018).

Emixustat hydrochloride is a small synthetic protein molecule which modulates the visual cycle. It is supposed to inhibit the RPE65 and thereby reduce the accumulation of toxic waste in the RPE cells (Bavik et al. 2015). In a controlled trial, emixustat hydrochloride given orally for 2 years failed to stop the annual growth rate of the GA lesions. Moreover, there were ocular adverse events like delayed dark adaptation in more than 50% and chromatopsia, erythropsia and visual impairment in 15–18% of patients (Rosenfeld et al. 2018).

5.4.7 New Approaches and Clinical Trials for AMD Gene Therapy

Eye, especially the subretinal space, being an immune-privileged site is ideally suited for gene therapy. The challenges are the size of the vector, its carrying capacity and its immunoreactivity. Presently the adeno-associated virus (AAV) in its various seroforms is the most commonly used vector. At least 18 ongoing trials are registered with the clinical trials registry in the USA to study the safety of gene therapy in phase I/II studies that have either been completed or still recruiting patients. Phase III trials are as yet far away but on the horizon (https://clinicaltrials.gov/ct2/results?cond=ARMD & term=Gene+therapy+OR+CRISPER & type= & rslt= & age_v= & gndr= & intr= & titles= & outc= & spons= & lead= & id= & cntry= & state= & city= & dist= & locn= & rsub= & strd_s= & strd_e= & prcd_s= & prcd_e= & sfpd_s= & sfpd_e= & rfpd_s= & rfpd_e= & lupd_s= & lupd_e= & sort=).

5.4.7.1 Choice of a Vector in Gene Therapy

The route of delivery of the vector into the eye has a bearing on its capacity to elicit an immune response and also its efficacy on the target cells. When delivered in the subretinal space, it is less immunoreactive (Li et al. 2008) but is limited by the risk of attendant surgical complications and the skills required for doing a pars plana vitreous surgery and raising a bleb under the retina to deliver the vector close to the macular area. The other routes being explored recently are intravitreal injections (IVT) and the suprachoroidal space (Kansara et al. 2020). When the vector is delivered via an IVT route, the internal limiting membrane of the retina offers resistance for the transduction of the gene; however, the new serotypes can overcome this challenge (Dalkara et al. 2009).

AAV is the most favoured vector at present. However, pre-existing antibodies to the wild-type AAV exist in the population from 72% to AAV2 to 47% to AAV8 serotypes (Boutin et al. 2010). Moreover, nearly one-quarter of the patients found negative for the AAV antibodies also were found to have transduction inhibition due to the presence of non-neutralizing antibody factors (Falese et al. 2017). This may be a major limiting factor in the transduction of the gene using this vector. To overcome the challenge of limitation of the gene size that can be carried by the viral vectors and repeated dosing if the need arises, non-viral vectors are being considered. While the gene size is not an issue with the non-viral vectors, the major challenge is transfection of the nucleus. Mechanical and chemical methods are being tested in the labs to improve the efficacy of the non-viral vectors delivered into the suprachoroidal space (Kansara et al. 2020).

Advances in polymer chemistry have led to the development of cationic poly amino esters that bind the negatively charged DNA and form nanoparticles which on entering the nucleus release the DNA. When such non-viral vectors are delivered in the suprachoroidal space, the genes are expressed over a much larger area of the retina (Shen et al. 2020).

5.4.7.2 Clinical Trials of Gene Therapy in Dry AMD

Nearly 90% of the AMD patients have a dry type of disease that shows progressive loss of RPE, choriocapillaris and the photoreceptors in the macular area (Figs. 5.7 and 5.8). As yet there is no known effective treatment for dry AMD. An overactive complement system is known to create a membrane attack complex, the MAC on the cell membrane that kills the cell. This self-destruction of the cell is blocked by a protein CD59. Can the overexpression of this gene increase the soluble CD69 factor and prevent this phenomenon? A study of an escalating dose of CD59 gene, AAVCAGsCD59 (HMR59), delivered as a single IVT injection tested by Hemera Biosciences in 17 patients with advanced dry AMD was completed in Dec 2019 (ClinicalTrials.gov Identifier: NCT03144999). The study is currently under extended follow-up to see long-term safety. A proof-of-concept study has also been initiated to recruit 25 patients with treatment-naive nAMD who would receive a single IVT injection of AAVCAGsCD59 on day 7 following an anti-VEGF injection at day 0. The anti-VEGF injections would be given as needed for 12 months and patients followed for 24 months. The study is expected to be completed by Jan 2022 (ClinicalTrials.gov Identifier: NCT03585556).

A study sponsored by Gyroscope Therapeutics (NCT 04437368) was fast-tracked in 2020 by the US FDA for testing two doses of GT005, a complement factor I (CFI) gene delivered subretinally in a randomized trial of 75 genetically defined patients with geographic atrophy (GA) of the macula for which no treatment exists so far. The study will see whether the GT005 is safe, well-tolerated and halts the progression of GA. The study is expected to be completed in Feb 2023.

Polymorphisms in the CFI gene are associated with non-functioning complement factor I leading to unbridled activation of the complement pathway and has been detected in patients with GA (Wang et al. 2016). Administration of GT005 in a mouse and non-human primate model showed a strong and sustained expression of CFI (Ellis et al. 2020).

5.4.7.3 Clinical Trials of Gene Therapy in nAMD

One of the earliest intraocular gene therapy trials used an IVT injection of AAV vector AdPEDF.11 expressing pigment epithelium-derived growth factor (PEDF) gene. This work showed the feasibility but only limited efficacy, as the effect on the nAMD did not last long but paved the way for future gene therapies. At least 25% of patients showed mild intraocular inflammation (Campochiaro et al. 2006).

A study, sponsored by Genzyme, a Sanofi Company, using single intravitreal injection of AAV2-sFLT01, a vector designed to expresses a gene to neutralize VEGF in patients with nAMD, has been ongoing since 2010 and was expected to be completed in 2018 (ClinicalTrials.gov Identifier: NCT01024998). Initial safety results were reported in 2017 (Heier et al. 2017).

A lentiviral equine infectious anaemia virus (EIAV) vector, Retinostat, coding for endostatin and angiostatin was injected subretinally in 21 patients with advanced nAMD and the trial completed in 2015 (ClinicalTrials.gov Identifier: NCT01301443). Results were published (Campochiaro et al. 2017a, b). Although the increased levels of endostatin and the angiostatin in the aqueous humour were

reproducible and the vector was tolerated well and found safe, the effect did not translate into any significant reduction in the leakage from the new vessels (Campochiaro et al. 2017a, b).

A one-time injection in suprachoroidal space of vector RGX-314 carrying a gene to neutralize VEGF is being tested in a multicentric trial by Regenxbio Inc. in patients with nAMD to reduce the burden of anti-VEGF injections (ClinicalTrials.gov Identifier: NCT04514653; NCT03066258). Highly encouraging results of the phase I/IIa were reported recently with significant improvement of visual acuity and reduction in retinal thickness with a significant reduction in the number of injections of ranibizumab, the anti-VEGF agent used in the study (http://pharmabiz.com/ArticleDetails.aspx?aid=130148&sid=2).

A recombinant gene therapy vector carrying a code for aflibercept, the ADVM-022 (AAV.7m8-aflibercept), is being tested by Adverum Biotechnologies, Inc. in 30 patients of nAMD in phase I trial and is expected to be completed by June 2022 (ClinicalTrials.gov Identifier: NCT03748784).

Most of the trials conducted so far have looked at the safety issues of the gene therapy and are not powered to test the long-term efficacy. While a recent 3-year follow-up study of phase I/II trial of a recombinant rAAV.sFLT-1 gene therapy established the use of this subretinal delivery platform in the elderly population, its biological efficacy in patients with advanced nAMD was not confirmed unequivocally (Rakoczy et al. 2019). Pre-existing immunity to the vector may have been responsible for the variable response seen in this study (Campochiaro et al. 2017a, b).

5.4.7.4 Optogenetics: Mutation-Independent Gene-Based Treatment of Retinal Diseases

Because of the common occurrence of heterogeneity in monogenic disorders and the frequent occurrence of complex polygenic diseases, many patients with retinal diseases are not good candidates for mutation-based therapies. For such patients focus is shifting towards mutation—independent therapies like the use of neuroprotective factors (NPF), miRNA, gene editing and optogenetics (Carrella et al. 2020). When all the photoreceptors have degenerated, the NPFs and the mutation-based gene replacement/enhancement therapies do not work. In these situations, optogenetics is a way forward to get the expression of photosensitive molecules in the retinal circuitry, especially the RGCs. Following the successful use of this approach in preclinical studies, phase I/II trials are already underway. Twelve patients are being recruited to assess Safety and Tolerability of Intravitreal RST-001 in Patients with RP (ClinicalTrials.gov Identifier: NCT02556736). Another study is enrolling 18 patients for phase1/2 open-label trial of single IVT injection of a gene encoding for a light-sensitive protein GS030-DP and a wearable optronic visual stimulation device, GS030-MD, in patients of non-syndromic RP (ClinicalTrials.gov Identifier: NCT03326336). The safety results in the first six patients were reported in 2020 [https://www.gensight-biologics.com/2020/04/14/gensight-biologics-announces-positive-data-safety-monitoring-board-review-of-pioneer-phase-i-ii-

trial-of-gs030-combining-gene-therapy-and-optogenetics-for-the-treatment-of-retinitis-pigmentosa/ accessed 2210.2020].

Achieving the intensity of light required to activate the animal-sourced photopigments is likely to be a major challenge as intense light itself may damage the retina further. Detailed mechanisms of how optogenetics can restore vision in the blind and how the challenges posed by this technology can be overcome have been reviewed recently (The Lasker/IRRF Initiative for Innovation in Vision Science 2014). In a breakthrough in the optogenetics technology, investigators succeeded in rescuing the vision of phenotypic deficit rd10 mice using a multifunctional opsin, MCO-1, that was delivered to the bipolar cells using an AAV2 vector restoring the vision circuitry (Batabyal et al. 2020). The protein was not expressed in any other cells except the bipolar cells and the effect was still present 6 months later. Unlike the previous opsins used, this opsin does not need intense light and gets activated even in ambient light (Batabyal et al. 2020). Bipolar cells are located downstream to the photoreceptors and participate in the transduction of visual impulses onwards to the visual cortex via the RGN fibres. In the annual meeting of the AAO November 2020, highly favourable results of the phase 1/2a trial were reported in 11 patients who received the gene vMCO-010 (Nanoscope) at the higher dose 3.5×10^{11} VG per eye. At 16 weeks, seven of the eight high-dose patients showed +15 letters improvement in visual acuity. Mild elevation of the IOP was noted in five patients and mild inflammation in three patients.

5.4.7.5 Glaucoma on the Threshold of Gene Therapy

Glaucoma is a chronic progressive disease that leads to blindness by progressive atrophy of the retinal nerve fibre layer (RNFL). One of the most common and effective strategies to control the progression of glaucoma has been to lower the intraocular pressure (IOP) either by drugs, lasers or incisional surgery. While gene editing using CAS9 has been used to correct a mutation in myocilin, a glaucoma gene, it may not be a viable approach in glaucoma which being a non-monogenic disease with hundreds of mutations will require targeting individual mutations. Targeting physiology of the aqueous humour production can be one of the alternate approaches. Aquaporins (Aqp) are a family of transmembrane water-transporting proteins. Transgenic mice lacking Aqp1 have lower IOP due to reduction in the aqueous production (Zhang et al. 2002). Wu et al. used an intravitreal injection of engineered ShH10 serotype AAV in a mouse model of glaucoma to deliver *S. aureus*-derived CRISPR-Cas9 to disrupt aquaporin1 proteins in the ciliary body and succeeded in reducing IOP (Wu et al. 2020). Till date, however, this strategy has not yet reached the stage of a clinical trial.

5.4.8 Stem Cell Therapy

Adult stem cells are present in several if not all organs and tissues, mainly for repair purposes, and the most abundant of these are found in the bone marrow for generating the circulating red and white blood cells. Bone marrow transplant or

haematopoietic stem cell transplants mainly for the leukaemias have been among the most successful transplants for more than 50 years. Stem cells are unique unspecialized cells that can divide indefinitely and can be induced to differentiate into specialized cells. Besides the bone marrow, the other major sources of stem cells in humans is embryonic stem cells (hESCs) sourced from the inner cell mass of the blastocyst from unused embryos. Use of hESC is as yet highly restricted because of far-reaching ethical issues. Moreover, these cells express HLA antigens and, if transplanted, would require lifelong immunosuppressive therapy. In 2006, a breakthrough in stem cell technology was reported by Takahashi and Yamanaka (2006) when they succeeded in inducing adult fibroblasts into inducible pluripotent cells (iPSC) by transduction of four transcription factors, namely, Oct3/4, Sox2, Klf4 and c-Myc first in mouse fibroblast and soon after in the human fibroblasts (Takahashi and Yamanaka 2006; Takahashi et al. 2007).

5.4.9 Cell-Based Therapy

The idea of replacing the dead retinal cells has been around for a long time and subject of extensive research in the labs; however, the challenge has been the non-integration of the transplanted cells into the retinal milieu. Muller cells are the main macroglia of the retina and stretch across the entire thickness of the neural retina. Besides providing the structural elasticity to the retina, these cells maintain very intimate contact with the photoreceptors, bipolar cells, horizontal cells, amacrine cells, RGC and the capillary endothelial cells and control glucose and glycogen metabolism and phototransduction, scavenge glutamate from the extracellular space and in general maintain homeostasis in the neural retina. More importantly, these cells recycle neurotrophic factors. Recently these cells have also been suggested to play a significant role in preventing oxidative damage. Photoreceptors derived from human Muller cells when transplanted in a rat model have restored rod function (Jayaram et al. 2014). Improvement of retinal function has been seen in the cat model of Muller cell-derived RGCs (Becker et al. 2016). Muller cells isolated from induced pluripotent stem cell-derived organoids when transplanted have shown improved functioning of the RGCs (Eastlake et al. 2019). Given the discovery that some human Muller cells also possess stem cell-like characteristics, in the future, it may be possible to renew retinal neurons using these cells (Eastlake et al. 2020).

5.4.10 Stem Cells in Translational Research

As the iPSC are pluripotent, they can be used for generating any retinal cell that has undergone degeneration. Human retinal organoid has been successfully created using iPSC (Zhong et al. 2014). The SC can be placed in the subretinal space as suspensions or sheets of cells grown on a thin template and the results of initial results of using hESC induced as RPE cells have been reported in ARMD and Stargardt's disease (Schwartz et al. 2015, 2016) (ClinicalTrials.gov, numbers

NCT01345006 (Stargardt's macular dystrophy) and NCT01344993 (age-related macular degeneration)). The major concern of using SC is its potential for unbridled proliferation and tumour formation. The authors did not report any adverse events related to the stem cells (Schwartz et al. 2016). Use of HiPSC-RPE from a 3-D organoid culture was shown to rescue the structure and function of photoreceptors after subretinal transplantation in a mouse model, and the PEDF was increased significantly (Zhu et al. 2020a, b). Several studies have completed trials on safety and tolerability of the use of hESC suspension in AMD or seeded on a substrate in Stargardt's macular degeneration (SMD) (ClinicalTrials.gov Identifier: NCT02903576), MA09-hRPE in patients with SMD (ClinicalTrials.gov Identifier: NCT01469832) and hESC-RPE in SMD (ClinicalTrials.gov Identifier: NCT02941991).

The major challenge of using either the hESC or the iPSC is the lack of biological competence of these cells, namely, the phagocytic properties and the anti-oxidative activity among other several biological functions these cells have to perform. One reason for this may be due to the short time in which these cells are grown in the lab (Qiu 2019). This has led to hyperpigmentation, a sign of accelerating ageing, in the transplanted RPE cells due to lack of antioxidant properties. The field of stem cells transplant is as yet in infancy (Qiu 2019).

Interim results of a human phase I/II trial of subretinal transplant of hESC-derived RPE cells (OpRegen) in advanced dry macular degeneration (NCT 02286089) revealed that OpRegen cells were well tolerated for 3 years and encouraging structural changes were noted (Data presented at the annual meeting of the AAO, Nov 2020).

In a breakthrough therapy of the first human retinal photoreceptor transplant in Japan, the induced pluripotent stem cells taken from the blood of a healthy donor were first grown into a 3-D retina (1 mm $\times$ 0.2 mm) and transplanted in the retina of the right eye of a patient who was blind from extensive retinal degeneration and could just perceive light or darkness. She would be watched for the next 6-months for the acceptance of the graft and whether she would see [https://english.kyodonews.net/news/2020/10/7c9e75ff0f1c-1st-ever-ips-visual-cell-transplant-performed-without-complications.html].

5.4.11 iPSC-TM Cells Transplant for Lowering of Intraocular Pressure (IOP) in Glaucoma

There is a constant flow of the aqueous humour from the posterior to the anterior chamber which finally drains out of the eye from the angle of the anterior chamber through a network of collagen fibres, the trabecular meshwork (TM) which is lined with specialized postmitotic cells (TM cells). The TM cells show an age-related decrease in the number and significantly more so in eyes with glaucoma (Alvarado et al. 1984; Rodrigues et al. 1976). In a proof-of-concept preclinical study of a novel approach, iPSCs differentiated into trabecular meshwork cells (TM cells) were successfully transplanted in the eyes of transgenic mice that led to control of IOP

and rescued the neurons (Zhu et al. 2016). In a further exploration of this approach, iPSC-TM transplanted in the perfusion-cultured human donor eyes led to endogenous proliferation of the TM cells (Zhu et al. 2020a, b).

5.4.12 iPSC to Populate the Corneal Endothelial Cells

Corneal transparency is maintained by an active pump located in a single layer of postmitotic endothelial cells that lines the posterior surface of the cornea. The endothelial cells not only prevent the aqueous humour to enter the corneal stroma but also remove the water ions from the corneal stroma. In healthy young people, the endothelial cells density is ~2500 cells/mm^2. Besides the age-related decrease (0.6%/year) in their numbers, trauma including cataract surgery, corneal infections and corneal dystrophies may cause substantial loss of these cells, and if they fall below a critical threshold (<400–500 cells/mm^2), corneal oedema ensues causing loss of vision. Till recently, the only treatment possible was a corneal transplant from a human donor eye. A highly efficient method has been developed to induce pluripotent stem cells to differentiate into corneal endothelial cells and form into a monolayer of endothelial cells paving way for a near-future replacement of corneal endothelium (Zhao and Afshari 2016; Price et al. 2020).

5.4.13 Use of Magnetic Nanoparticle-Labelled Cultured Human Endothelial Cells

In November 2020 annual meeting of the AAO, Dr Jeffery L. Goldberg presented results of a novel technique wherein patients were injected with magnetic nanoparticle-labelled cultured human endothelial cells into the anterior chamber of the eye and made to wear magnetic patches over the eye to ensure uniform distribution of the endothelial cells over the back of the cornea. Fourteen of the 21 patients thus treated showed significant reduction in corneal thickness and nine had at least three-line improvement in visual acuity (unpublished data).

5.5 Discovery of Cytokines and Era of Biological Therapy in Ocular Inflammations

The discovery of molecules that blocked or interfered with the viral replication in the infected cells, by Alick Isaacs and Jean Lindenmann (Isaacs and Lindenmann 1957; Isaacs et al. 1957; Burke 2009) in 1957 and hence called interferons, was followed over the years by the detection of a variety of other small molecules broadly known as cytokines. They primarily include the interleukins first named so in 1979 (di Giovine and Duff 1990). These discoveries heralded the era of gaining insight into the orchestra of cell biology, more specifically how these small protein molecules, which cannot enter a cell but bind with the specific receptors on the

cell surface, dictate activation of the intracellular signalling pathways to determine how the cells interacted, got activated, produced cytokines, differentiated, proliferated or died. They essentially control and regulate the functioning of a variety of cells under stress and thereby play a critical role in embryogenesis, haematopoiesis, inflammation and both innate and adaptive immune mechanisms. The cytokines although have a short half-life, these are released in cascades in response to cellular stress. The cytokines are redundant as several cell types can produce these molecules and show pleiotropy as a specific cytokine can bind with a variety of cells. The largest family among the cytokines is the interleukin (IL) family, others being interferons, tumour necrosis factor (TNF) and chemokines. Briefly, IL-1 is produced by macrophages and B cells and stimulates T-helper cells and plays a role in inflammation; IL-2 is produced by the Th1 cells and causes proliferation and differentiation of T cells; IL-6 is produced by a variety of cells such as B cells, Th2, macrophages and endothelial cells and helps in differentiation of B cells into plasma cells and antibody production and is a key regulator of inflammation; IL-17 is secreted by the Th17 cells. TNF-α, an acute phase reactant, is produced by macrophages, monocytes, lymphocytes and granulocytes and is responsible for acute inflammation (Ferreira et al. 2019).

In their functions cytokines have either synergistic or antagonistic (blocking) actions. Cytokines IL-1 and TNF-α are proinflammatory and acting in synergy make the inflammation worse and cause tissue destruction. During acute infection, there is a storm of proinflammatory cytokines, but as the infection gets controlled, the genes expressing the cytokines shut down. On the contrary, in autoimmune disorders, a dysregulation of the cytokine gene expression due to genetic predisposition is responsible for the persistence of activated cells.

Once the functional biology of the cytokines was known and it was possible to clone them by cDNA and recombinant technology, a new era of biological therapy dawned (Dinarello 2007).

Uveitis is of two types, infectious and non-infectious, the latter is the most common cause of autoimmune inflammation. Untreated uveitis leads to tissue destruction and blindness. Since the 1950s, the corticosteroids have been the standard of care in the treatment of uveitis and although they control inflammation very efficiently and effectively, but only at the cost of undesirable ocular and systemic side effects. Glucocorticoid receptors are present in almost all human cells and tissues and are responsible for the undesirable pleiotropic effects. To reduce the maintenance dose of corticosteroids, often these are combined with immunosuppressive agents that in general suppress the proliferation of all immune cells including the regulatory T cells (Tregs), the cells that regulate and control inflammatory response to auto or foreign antigens. Moreover, these also have their toxicity.

5.5.1 Development of Blocking Antibodies to Cytokines

There have been several strategies to block these cytokines by using antibodies, soluble receptors, receptor antagonists and protease inhibitors that have been greatly

successful in the treatment of rheumatoid arthritis (RA) and inflammatory bowel disease (Dinarello 2000). In the early 1980s, soluble monoclonal antibodies to the TNF-α, the cytokine that targets the pathways of inflammation and tissue repair, were used in patients with rheumatoid arthritis, a disabling autoimmune disorder of the joints (Dinarello 2007).

Among all the cytokines, TNF-α, IL-1β, IL-6 and IL-17 are the key mediators of endogenous inflammation of the eye, called uveitis, and have been found in the serum and ocular fluids of uveitis patients (Jawad et al. 2013; Kuiper et al. 2011; Ooi et al. 2006). Biological agents that target these cytokines or their receptors have been successfully used, and many of them have been US FDA approved in systemic inflammatory diseases. Although most of these are not approved by the FDA for treatment of uveitis, these have found widespread use for patients with uveitis who have associated systemic inflammatory disease (Reddy et al. 2018, 2019).

5.5.2 TNF-α Blocking Monoclonal Antibodies

Infliximab was the first chimeric antibody to TNF-α which blocks both the soluble and the membrane-bound TNF-α. It was developed to treat IBD and RA. Several studies reported dramatic remission of inflammation in patients with autoimmune uveitis, HLA B27-associated acute anterior uveitis, Behcet's disease, sarcoidosis and JIA-associated uveitis (Benitez-del-Castillo et al. 2005; Kahn et al. 2006; Suhler et al. 2005; Ardoin et al. 2007; Lopez-Gonzalez et al. 2009).

Adalimumab was the first fully human IgG1 antibody to TNF-α and blocks the TNF-α from binding to its receptor. It was FDA approved for the treatment of RA, JIA, IBD and ankylosing spondylitis. A post hoc analysis of two parallel trials on the use of adalimumab found significant patient-reported visual improvement in non-infectious uveitis (Sheppard et al. 2017). In a multicentric trial of infliximab versus adalimumab for refractory uveitis that included Behcet's disease, JIA-associated uveitis, spondyloarthropathy and sarcoidosis, the response rate was 87% at 6 months and 93% at 12 months (Vallet et al. 2016). In 2016, adalimumab was FDA approved for the treatment of intermediate, posterior and panuveitis. The TNF-α blocking drugs are not without serious side effects that include reactivation of tuberculosis, demyelinating disease multiple sclerosis, sarcoidosis, Guillain-Barre syndrome, etc. (Vallet et al. 2016).

A systematic review of anti-TNF therapies showed that nearly 2.2% of patients had to discontinue therapy because of side effects (Cordero-Coma et al. 2013).

A multicentric randomized placebo-controlled adalimumab for JIA-associated uveitis stopping trial (ClinicalTrials.gov Identifier: NCT03816397) is recruiting 118 patients and likely to be completed in Feb 2023.

Certolizumab pegol, an anti-TNF pegylated antibody lacking the fc segment, has been found effective in controlling the articular manifestations of axial spondyloarthropathy. In the RAPID-axSpA, a 204-week trial, certolizumab was effective in lowering the recurrence rates of acute anterior uveitis in patients suffering from spondyloarthropathy (Rudwaleit et al. 2016).

Non-viral ocular gene therapy for controlling intraocular inflammation: While systemic inhibitors are FDA approved, there is no approved platform for local delivery of anti-TNF agents. A non-viral gene therapy using plasmid electrotransfer of pEYS606, coding for a fusion protein consisting of an extracellular domain of the soluble p55 TNF-α receptor and human IgG1 Fc domain, into the ciliary body for sustained expression of the protein has been successful in the preclinical studies and is undergoing a phase I/II trial (ClinicalTrials.gov Identifier: NCT03308045) in 29 patients with non-infectious uveitis (Touchard et al. 2018).

5.5.3 Gevokizumab-Anti-IL-1β Antibody

IL-1β is a strong mediator of inflammation and its serum levels are elevated in several autoimmune and inflammatory diseases. Gevokizumab was developed as a potent humanized neutralizing antibody to IL-1β to control local inflammation by a convenient systemic administration (Owyang et al. 2011). Although it caused a rapid resolution of inflammatory activity in Behcet's disease in phase I and II studies, in a multicentric open-label placebo-controlled trial (EYEGUARD B-ClinicalTrials.gov NCT 01965145), it was not found superior in preventing the occurrence of ocular exacerbations in Behcet's disease (Tugal-Tutkun et al. 2018).

5.5.4 Tocilizumab-IL-6 Antibody

IL-6, a potent mediator of inflammation, is a very good target for controlling inflammation. Tocilizumab was the first IL-6 antibody that was developed to bind both soluble and membrane-bound IL-6. It is approved by the US FDA for the treatment of RA and JIA. In the STOP-uveitis study, intravenous tocilizumab (4 mg/kg and 8 mg/kg) was found effective in improving posterior segment inflammation in non-infectious uveitis and has a good safety profile (Sadiq et al. 2020; Karkhur et al. 2019). Several other molecules targeting IL-6 are in the pipeline (Karkhur et al. 2019).

5.5.5 Secukinumab-IL-17A Antibody

Secukinumab, a fully humanized antibody to IL-17A, is FDA approved for the treatment of psoriasis and can be delivered both intravenously and subcutaneously. There are however conflicting results of its efficacy in reducing the recurrence rates of non-infectious uveitis. Inadequate bioavailability of the subcutaneous drug may have affected the efficacy of the drug (Hueber et al. 2010; Dick et al. 2013; Letko et al. 2015).

5.5.5.1 Other Biological Agents

Some other biological molecules such as TNF-α inhibitor Golimumab, IL-1 inhibitor Canakinumab, IL-2 inhibitor Daclizumab, Janus kinase-1 inhibitor Filgotinib from the family of tyrosine kinase inhibitor have shown promising results in initial clinical studies (Hassan et al. 2019; Miserocchi et al. 2020; Orrock and Ilowite 2016; Reddy et al. 2019). There is a huge unmet demand for safe and effective therapies for autoimmune uveitis that should not only control inflammation effectively but also prevent recurrences. Recurrences of inflammation on the withdrawal of the drug are very common and remain the most important challenge. Many of these drugs need to be given intravenously requiring hospitalization, adding to the burden in patient care. Another goal is therefore convenient drug delivery. As our knowledge of the inflammatory pathways expands, newer potential sites are located to block inflammation. Many promising agents appear on the horizon for a while and undergo phase I and II trials but fail to meet the end point of safety and efficacy in phase III trials. Many of these biological drugs that were found useful in systemic inflammations were either not effective or did not meet the benchmark of the risk-benefit ratio.

5.5.5.2 Rho Kinase Inhibitors in Preserving Corneal Endothelium and Glaucoma Treatment

Phosphorylation of proteins, a reversible process mediated by protein kinases in which the amino acid residues covalently bind to a phosphate group, plays a key regulatory role in cell biology in activating/inactivating of various enzymes and receptors, cell proliferation. Protein kinases play a key role in tumour formation and hence form a good therapeutic target. Many protein kinase inhibitors are approved for cancer management (Ardito et al. 2017). Rho kinase (ROCK) is a type of protein kinase mainly involved in cytoskeleton and shape of the cell and prevent apoptosis. ROCK inhibitors have been used as eye drops as well as an injection into the anterior chamber to prevent further corneal endothelial cells loss in patients with Fuchs' corneal dystrophy undergoing cataract surgery (Okumura et al. 2017). They have also been used as an adjunct along with the injection of cultured endothelial cells into the anterior chamber. Topical ripasudil hydrochloride hydrate 0.4%, a potent ROCK inhibitor, is being tested six times a day for 2 weeks, in a placebo-controlled double-blind trial in 72 patients for its potential benefits in patients after Descemet membrane endothelial keratoplasty for Fuchs' endothelial dystrophy (ClinicalTrials.gov Identifier: NCT03813056).

ROCK inhibitors have also been used in the treatment of glaucoma as these drugs lower IOP by increasing the aqueous outflow facility (Tanihara et al. 2013, 2015; Tanna and Johnson 2018). Two drugs, netarsudil and ripasudil, have been FDA approved as glaucoma therapy. Physiology of Rho inhibitors and their applications in ophthalmology was reviewed recently (Moura-Coelho et al. 2019).

5.6 Medical Diagnostics in Ophthalmology

Bench research discoveries in the last 2–3 decades like the polymerase chain reaction, the next-generation sequencing and proteomics has brought about a paradigm shift in the management of patients in the ophthalmology clinics all over the world.

5.6.1 Polymerase Chain Reaction

A simple technique, to multiply the DNA a billion fold in a short time, the polymerase chain reaction (PCR) was invented by a US chemist Kary Banks Mullis in 1983, more than 100 years after the discovery of DNA and 30 years after the discovery of the double-helix structure of DNA by Francis Crick and James Watson in 1953. The technique requires the DNA to be copied (the template DNA), two small strands of oligonucleotides (target DNA primers) and the four nucleotides and a heat-stable Taq polymerase (sourced from a heat-stable bacteria, *Thermus aquaticus*). The mix is heated and cooled in cycles to allow the heat to first break the H+ bonds of the template DNA and split it into two strands, and when cooled the primers attach to each strand of the template to their complementary sites (annealing) followed by extension during which the nucleotides attach on either side of the primers to the template and resulting in two copies of the original DNA. The cycle, each lasting a few minutes, is repeated and a billion copies of the target DNA can be generated in 30 cycles (https://www.britannica.com/biography/Kary-Mullis).

5.6.2 PCR to Diagnose Ocular Infections

The technique brought about a stupendous revolution in biology research as well as in the medical diagnostics. The conventional technique of diagnosing infectious organisms such as the bacteria, the viruses, the fungi and the parasites required their culture on special media, techniques, although gold standard and highly specific, are time-consuming and not highly sensitive. By using PCR, infections can be diagnosed even if a very tiny amount of the DNA of the offending organism is present in the patient's fluid or tissue sample. In less than 10 years of its development in the research lab, the technique found its widespread use in the clinical microbiology laboratories world over to diagnose viral infections of the retina caused by HSV, VZV and CMV with more than 95% sensitivity and nearly 100% specificity. Necrotizing infections of the retina caused by different viruses, toxoplasmosis, a parasite and syphilis, a bacterial infection may clinically look similar especially in immunosuppressed individuals. Diagnostic PCR in these cases made it possible to reach a very precise diagnosis of the offending viruses (Fox et al. 1991; Fenner et al. 1991; Nishi et al. 1992; Biswas et al. 1993; McCann et al. 1995; Short et al. 1997; Knox et al. 1998). It is especially of use in patients who present with the atypical clinical picture (Montoya et al. 1999). More recently, using PCR, Ebola virus was

found persisting in the aqueous humour of a patient who had recovered from the Ebola virus infection (Varkey et al. 2015).

In a major development, a highly sensitive and specific strip multiplex-PCR test has been developed to simultaneously test for 24 common infections of the eye which yielded results equivalent to the more conventional quantitative PCR (Nakano et al. 2020).

5.6.3 Challenge of Diagnosing Intraocular Tuberculosis

Inflammation of the various ocular tissues especially the uvea and retinal vessels caused by *Mycobacterium tuberculosis* (MTB) had been suspected for more than 100 years, but due to the paucibacillary nature of the MTB infection in the eye and a predominant immune response, it remained a challenge to detect the MTB from the ocular tissues without sacrificing the eye. Using PCR it was demonstrated by the author that patients suspected of MTB infections could be successfully treated when diagnosed by PCR (Gupta et al. 1998). Compared to the viral infections, PCR is less sensitive in detecting intraocular infections caused by MTB leading to a number of modifications in the technique to improve the sensitivity of the PCR including the nested PCR, multitarget PCR (amplification of multiple target genes of the same organism) and multiplex PCR (amplification of target genes of multiple organisms) and the real-time quantitative PCR (Therese et al. 2005; Sharma et al. 2013; Kumar et al. 2017; Singh et al. 2012). Although PCR has proven to be a suboptimal technique to diagnose MTB uveitis, and a negative PCR for MTB does not rule out the TB infection in the eye, MTB positive PCR helped in identification of many phenotypes of MTB uveitis which are now recognized all over the world including in those parts of the world that at present are non-endemic for MTB infections. Blindness resulting from the MTB infection of the eye can now be prevented (Gupta et al. 2001, 2003, 2005; Gupta and Gupta 2005). For the first time, using gene sequencing, it became possible to identify rifampicin-resistant MTB organisms infecting the eye that led to successful outcomes following specific treatment strategies (Sharma et al. 2014, 2015, 2019).

5.6.4 Next-Generation Sequencing

Ever since the discovery of the DNA/RNA structure and the PCR technique to multiply oligonucleotide sequences a billion times, a need was felt to know the exact base sequence of the nucleotides that constitute a gene and finally sequence the entire human genome. The first generation of sequencing was done by Sanger in 1977 by using 2′,3′dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphate as specific chain-terminating inhibitors of the DNA polymerase (Sanger et al. 1977; Kulski 2016). The Sanger technique remained in use for nearly three decades. It was extremely slow and expensive. The NGS currently in use are high-throughput massively parallel systems of sequencing that generate gigabase (Gb)-

sized sequences in days to hours at much-reduced cost (Kulski 2016). NGS sequences in parallel millions of small DNA fragments of 100–500 base length. In a human sample, most of it would consist of the human DNA but 0.1–8% would be derived from the offending organism. One of the challenges is to avoid contamination of the sample by non-pathogenic organisms during the sample collection. The sequence obtained is compared with the reference data for the diagnosis. An overabundance of a specific microbial DNA would be considered the infective organism. Moreover, sequences that indicate an antimicrobial resistance can also be known (Ma et al. 2019; Li et al. 2018). Interpretation of the massive data generated led to the development of the discipline of bioinformatics and 'omics' techniques such as genomics, transcriptomics, proteomics, metabolomics and microbiomics that have brought about a revolution in molecular medicine and cell biology. Integration of the number of omics can provide insight not only in the identification of the genetic variants and the pathogenesis mechanism of the diseases but also help in discovering the therapeutic targets (Hasin et al. 2017).

5.6.5 Next-Generation Sequencing to Diagnose Ocular Infections

Corneal infections are commonplace and result in corneal scarring and blindness from the eye and are the fourth common cause of blindness in the world. Endophthalmitis following cataract surgery, although uncommon, is one of the most devastating complications of the surgery. In more than 50% of the cases, the conventional smear and cultures fail to identify the infective organism. Next-generation sequencing is a relatively new technology for accurate detection of the pathogens both known and yet undiscovered that are responsible for eye infections so that patients can be treated with very specific therapy.

Using conventional techniques, including the cultures and PCR, many at time it is impossible to identify the microorganisms that gain entry into the eye during a diseased state. Negative reports do not rule out a microbial presence (Kirstahler et al. 2018). In patients of endophthalmitis, the NGS could demonstrate the presence of microbes in nearly 88% of vitreous samples compared to 44% using culture techniques (Deshmukh et al. 2019).

Using a metagenomics technique, the ocular microbiome of the vitreous fluid in normal controls and patients with post-fever retinitis reported a dysbiosis in the vitreous microbiome (Arunasri et al. 2020). The technology has a huge potential for its application in the field of ophthalmology as evidenced by the preliminary clinical reports.

5.7 Molecular Biomarkers

5.7.1 Biomarkers

Molecular biomarkers are markers that are present in the body tissues or fluids in health and the diseased state and consist mostly of proteins as genes, gene products, enzymes, antibodies or even cells. Biomarkers have to be detectable and measurable to be of help in the clinic in understanding the pathogenic mechanisms, help in making a diagnosis or prognosis of the disease and additionally serve as therapeutic targets.

5.7.2 Proteomics

Proteomics, as it relates to the eye, is the study of the identification and profiling of proteins in ocular fluids in a diseased and healthy state of the eye by mass spectroscopy using high-throughput technology. Most of the pathogenic mechanisms underlying the eye diseases are not known as yet. In recent years, protein profiling of the tear fluid, aqueous and vitreous humour in various diseases of the eye has been engaging the attention of researchers. Because of its great promise in understanding the biological pathways of diseases, ocular proteomics has recently become a component of the biology/disease-driven human proteomics project (B/D-HPP). The authors presented a catalogue of 4842 non-redundant proteins that were identified in the ocular tissues and fluids till 2013 (Semba et al. 2013). Agarwal et al. reviewed cytokines and biologics in non-infectious uveitis that have translational value in clinical practice (Agrawal et al. 2014). In addition to the ultrafiltrates from the blood, proteins from the RPE, photoreceptors and choroid are deposited in the vitreous. Studying proteomics from sites closer to the site of the diseased tissue/organ is more reliable than from the serum (Velez and Mahajan 2020).

5.7.3 Proteomics from Intraocular Fluids to Manage Rare Ocular Diseases

Conventional treatment is not effective in preventing blindness in a rare hereditary blinding autoinflammatory disease, the neovascular inflammatory vitreoretinopathy (NIV) caused by mutations in the CAPN5 gene (Velez et al. 2017). The proteomics profiling of ocular fluids led to the identification of superior and inferior targets for therapy. The cytokine panel in this disease revealed the presence of normal levels of TNF-α and absence of corticosteroid-sensitive pathways explaining the failure of the conventional treatment given to these patients (Velez et al. 2017). In a three-pronged approach including the anti-VEGF injections for the upregulated VEGF that led to vitreous haemorrhage in the patients rather than surgery, methotrexate was used instead of corticosteroids to resolve the inflammation, and the anti-IL-6 therapy

successfully prevented the development of fibrosis in these patients (Velez et al. 2017).

Using a mass spectrometry technique, SELDI-ToF, transthyretin (TTR) was found in aqueous humour as a potential biomarker for JIA-associated uveitis and other silent uveitis entities (KalininaAyuso et al. 2013).

Velez et al. (2016) detected many signature cytokines from the vitreous of patients with posterior uveitis including IL-23, TIMP-1 and TIMP-2 (IL-23), IL-1 receptor I, IL-17R, insulin-like growth factor-binding protein 2 (IGFBP-2), etc.

In a plasma metabolomics study of VKH disease, D-mannose, stearic acid and L-lysine were found as potential biomarkers of active VKH disease (Chen et al. 2020).

In a proteomics study of three patients with intermediate uveitis, a chronic bilateral recurrent inflammation of the vitreous humour, the upregulation of IL-23 was seen as a potential target for biological therapy (Sepah et al. 2020).

Primary vitreoretinal lymphoma (PVRL) is a rare intraocular malignancy which often masquerades as uveitis thereby delaying the diagnosis. High IL-10/IL-6 ratio and IL-1-RA levels were seen in nine of the ten patients with PVRL and not in other non-PVRL inflammations. Other markers that were found high in PVRL were CD 19, a pan B cell marker, two chemokines-monocyte chemotactic protein-1 and macrophage inflammatory protein-1-β and a skewed ratio of SmIgK/SmIgL (de Hoog et al. 2019). Fukunaga et al. (2020) found specific and likely useful biomarkers of inflammation in the vitreous in different aetiologies of intraocular inflammation. In patients with bacterial endophthalmitis, they found elevated levels of IL-6, IL-17A, G-CSF and IL-22; interferon-2α and RANTES in viral acute retinal necrosis; and IL-10 in primary vitreoretinal lymphoma. Proteomics analysis of the vitreous obtained by vitreous biopsy is a highly promising strategy in precision medicine in treating complex vitreoretinal diseases (Velez and Mahajan 2020).

5.7.4 Proteomics from Tears

Tears, secreted by the lacrimal and accessory lacrimal glands, are a complex mixture of water, electrolytes, lipids and a variety of proteins that forms a film over the corneal and the conjunctival surface. Because of the ease of access and collection, proteomics from the tears has been explored for several years not only in ocular disease but also some of the systemic disease (Pieragostino et al. 2015). The most reliable source of biomarkers is vitreous followed by the aqueous. The question remains whether the tears provide a reliable measure of cytokine activity comparable to the intraocular fluids. One of the questions the clinicians face when starting treatment in a patient with uveitis is whether the patient would respond to the conventional treatment. Elevated levels of IL-6 and IL-10 in tears were found in patients with uveitis at the baseline, which, however, did not correlate with the level of inflammation in the eye. But the tear IL-6 levels correlated with longer duration of inflammation whereas the elevated tear levels of IL-10 at baseline predicted response to treatment at 4 and 8 weeks (Shirinsky et al. 2020).

In a pilot study of JIA-associated uveitis and idiopathic chronic anterior uveitis, tears from JIA uveitis contained a similar cytokine profile as those reported from the aqueous, and more importantly these were similar to the ones found in arthritis (Angeles-Han et al. 2018). Proteomics of the tear fluid is being explored not only to diagnose intraocular inflammations but also to evaluate response to therapy in several systemic disorders.

References

Abelson MB, Smith L, McLaughlin J (2020) Two decades in translation. Rev Ophthalmol. https://www.reviewofophthalmology.com/article/two-decades-in-translation. Accessed 7 Oct 2020

Agrawal R, Iyer J, Connolly J, Iwata D, Teoh S (2014) Cytokines and Biologics in non-infectious autoimmune uveitis: bench to bedside. Indian J Ophthalmol 62(1):74–81. https://doi.org/10.4103/0301-4738.126187. PMID: 24492505; PMCID: PMC3955074

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE et al (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331(22):1480–1487. https://doi.org/10.1056/NEJM199412013312203. PMID: 7526212

Almeida RD, Manadas BJ, Melo CV et al (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and Pl-3 kinase pathways. Cell Death Differ 12:1329–1343

Alvarado J, Murphy C, Juster R (1984) Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. Ophthalmology 91(6):564–579. https://doi.org/10.1016/s0161-6420(84)34248-8. PMID: 6462622

Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, Hauser MA, Rickman CB, Bok D, Hageman GS, Johnson LV (2010) The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. Prog Retin Eye Res 29(2):95–112. https://doi.org/10.1016/j.preteyeres.2009.11.003. Epub 2009 Dec 2. PMID: 19961953; PMCID: PMC3641842

Angeles-Han ST, Yeh S, Patel P et al (2018) Discovery of tear biomarkers in children with chronic non-infectious anterior uveitis: a pilot study. J Ophthalmic Inflamm Infect 8(1):17. https://doi.org/10.1186/s12348-018-0156-5. Published 2018 Oct 16

Apte RS, Chen DS, Ferrara N (2019) Vegf in signaling and disease: beyond discovery and development. Cell 176:1248–1264

Ardito F, Giuliani M, Perrone D, Troiano G, Lo Muzio L (2017) The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). Int J Mol Med 40(2):271–280. https://doi.org/10.3892/ijmm.2017.3036. Epub 2017 Jun 22. PMID: 28656226; PMCID: PMC5500920

Ardoin SP, Kredich D, Rabinovich E, Schanberg LE, Jaffe GJ (2007) Infliximab to treat chronic noninfectious uveitis in children: retrospective case series with long-term follow-up. Am J Ophthalmol 144(6):844–849. https://doi.org/10.1016/j.ajo.2007.08.018

Arevalo JF, Fromow-Guerra J, Quiroz-Mercado H, Sanchez JG, Wu L, Maia M, Berrocal MH, Solis-Vivanco A, Farah ME, For the Pan-American Collaborative Retina Study Group (2007) Ophthalmology 114:743–750. https://doi.org/10.1016/j.ophtha.2006.12.028

Arunasri K, Mahesh M, Sai Prashanthi G, Jayasudha R, Kalyana Chakravarthy S, Tyagi M, Pappuru RR, Shivaji S (2020) Comparison of the vitreous fluid bacterial microbiomes between individuals with post fever retinitis and healthy controls. Microorganisms 8(5):751. https://doi.org/10.3390/microorganisms8050751. PMID: 32429503; PMCID: PMC7285296

Batabyal S, Gajjeraman S, Pradhan S et al (2020) Sensitization of ON-bipolar cells with ambient light activatable multi-characteristic opsin rescues vision in mice. Gene Ther 28:162–176. https://doi.org/10.1038/s41434-020-00200-2

Bavik C, Henry SH, Zhang Y, Mitts K, McGinn T, Budzynski E, Pashko A, Lieu KL, Zhong S, Blumberg B, Kuksa V, Orme M, Scott I, Fawzi A, Kubota R (2015) Visual cycle modulation as an approach toward preservation of retinal integrity. PLoS One 10(5):e0124940. https://doi.org/10.1371/journal.pone.0124940. PMID: 25970164; PMCID: PMC4430241

Becker S, Eastlake K, Jayaram H, Jones MF, Brown RA, McLellan GJ, McLellan GJ, Charteris DG, Khaw PT, Limb GA (2016) Allogeneic transplantation of Muller-derived retinal ganglion cells improves retinal function in a feline model of ganglion cell depletion. Stem Cells Transl Med 5(2):192–205. https://doi.org/10.5966/sctm.2015-0125

Benitez-del-Castillo JM, Martinez-de-la-Casa JM, Pato-Cour E, Méndez-Fernández R, López-Abad C, Matilla M, Garcia-Sanchez J (2005) Long-term treatment of refractory posterior uveitis with anti-TNFalpha (infliximab). Eye (Lond) 19(8):841–845. https://doi.org/10.1038/sj.eye.6701689. PMID: 15389273

Biswas J, Mayr AJ, Martin WJ, Rao NA (1993) Detection of human cytomegalovirus in ocular tissue by polymerase chain reaction and in situ hybridization. Graefes Arch Clin Exp Ophthalmol 231:66–70

Blanke ML, Van Dongen AMJ (2009) Chapter 13: Activation mechanisms of the NMDA receptor. In: Van Dongen AM (ed) Biology of the NMDA receptor. CRC Press/Taylor & Francis, Boca Raton. https://www.ncbi.nlm.nih.gov/books/NBK5274/

Boia R, Ruzafa N, Aires ID, Pereiro X, Ambrósio AF, Vecino E, Santiago AR (2020) Neuroprotective strategies for retinal ganglion cell degeneration: current status and challenges ahead. Int J Mol Sci 21(7):2262. https://doi.org/10.3390/ijms21072262. PMID: 32218163; PMCID: PMC7177277

Bonini S, Lambiase A, Rama P et al (2018) Phase II randomized, double masked, vehicle controlled trial of recombinant human nerve growth factor for neurotrophic keratitis. Ophthalmology 125: 1332–1343

Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, Masurier C (2010) Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther 21(6):704–712. https://doi.org/10.1089/hum.2009.182. PMID: 20095819

Burke DC (2009). http://www.brainimmune.com/the-discovery-of-interferon-the-first-cytokine-by-alick-isaacs-and-jean-lindenmann-in-1957/

Burnight ER, Wiley LA, Drack AV, Braun TA, Anfinson KR, Kaalberg EE, Halder JA, Affatigato LM, Mullins RF, Stone EM, Tucker BA (2014) CEP290 gene transfer rescues Leber congenital amaurosis cellular phenotype. Gene Ther 21(7):662–672. https://doi.org/10.1038/gt.2014.39. Epub 2014 May 8. PMID: 24807808; PMCID: PMC4188442

Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, Frank RN, Saperstein DA, Gupta A, Stout JT, Macko J, DiBartolomeo R, Wei LL (2006) Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Hum Gene Ther 17(2):167–176. https://doi.org/10.1089/hum.2006.17.167. PMID: 16454650

Campochiaro PA, Heier JS, Kherani S, Le-Halpere A, Scaria A (2017a) Ocular gene therapy for neovascular AMD: a new era?—Authors' reply. Lancet 390(10108):2140. https://doi.org/10.1016/S0140-6736(17)32424-8. Epub 2017 Nov 9. PMID: 29143750

Campochiaro PA, Lauer AK, Sohn EH et al (2017b) Lentiviral vector gene transfer of endostatin/angiostatin for macular degeneration (GEM) study. Hum Gene Ther 28(1):99–111. https://doi.org/10.1089/hum.2016.117

Carrella S, Indrieri A, Franco B, Banfi S (2020) Mutation-independent therapies for retinal diseases: focus on gene-based approaches. Front Neurosci 14:588234. https://doi.org/10.3389/fnins.2020.588234

Chawla B (2018) Bench to bedside: translational research demystified. Delhi J Ophthalmol 29(1): 4–5. https://doi.org/10.7869/djo.367

Chen L, Chang R, Pan S, Xu J, Cao Q, Su G, Zhou C, Kijlstra A, Yang P (2020) Plasma metabolomics study of Vogt-Koyanagi-Harada disease identifies potential diagnostic

biomarkers. Exp Eye Res 196:108070. https://doi.org/10.1016/j.exer.2020.108070. Epub 2020 May 18. PMID: 32439397

Chew EY (2020) Age-related macular degeneration: nutrition, genes and deep learning—the LXXVI Edward Jackson Memorial lecture. Am J Ophthalmol 217:335–347. https://doi.org/10.1016/j.ajo.2020.05.042

Chew EY, Clemons TE, Jaffe GJ et al (2019) Effect of ciliary neurotrophic factor on retinal neurodegeneration in patients with macular telangiectasia type 2. A randomized clinical trial. Ophthalmology 126(4):540–549. https://doi.org/10.1016/j.ophtha.2018.09.041

Cho GY, Schaefer KA, Bassuk AG, Tsang SH, Mahajan VB (2018) CRISPR genome surgery in the retina in light of off-targeting. Retina 38(8):1443–1455. https://doi.org/10.1097/IAE.0000000000002197. PMID: 29746416; PMCID: PMC6054556

Connolly DT, Olander JV, Heuvelman D, Nelson R, Monsell R, Siegel N, Haymore BL, Leimgruber R, Feder J (1989) Human vascular permeability factor. Isolation from U937 cells. J Biol Chem 264:20017–20024

Contopoulos-Ioannidis DG, Ntzani EE, Ioannidis JPA (2003) Translation of highly promising basic science research into clinical applications. Am J Med 114:477–484

Cordero-Coma M, Yilmaz T, Onal S (2013) Systematic review of anti-tumor necrosis factor-alpha therapy for treatment of immune-mediated uveitis. Ocul Immunol Inflamm 21(1):19–27. https://doi.org/10.3109/09273948.2012.723107. PMID: 23323577

Cunningham ET Jr, Adamis AP, Altaweel M, Aiello LP, Bressler NM, D'Amico DJ, Goldbaum M, Guyer DR, Katz B, Patel M, Schwartz SD, Macugen Diabetic Retinopathy Study Group (2005) A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular edema. Ophthalmology 112(10):1747–1757. https://doi.org/10.1016/j.ophtha.2005.06.007. PMID: 16154196

Daiger SP, Sullivan LS, Bowne SJ (2013) Genes and mutations causing retinitis pigmentosa. Clin Genet 84(2):132–141. https://doi.org/10.1111/cge.12203

Dalkara D, Kolstad KD, Caporale N et al (2009) Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. Mol Ther 17:2096–2102

De Falco S (2012) The discovery of placenta growth factor and its biological activity. Exp Mol Med 44(1):1–9. https://doi.org/10.3858/emm.2012.44.1.025. PMID: 22228176; PMCID: PMC3277892

de Hoog J, Dik WA, Lu L, Heezen KC, Ten Berge JC, Swagemakers SMA, van der Spek PJ, van Dongen JJM, van der Velden VHJ, Rothova A, Langerak AW (2019) Combined cellular and soluble mediator analysis for improved diagnosis of vitreoretinal lymphoma. Acta Ophthalmol 97(6):626–632. https://doi.org/10.1111/aos.14036. Epub 2019 Jan 27. PMID: 30688042; PMCID: PMC6796208

Deshmukh D, Joseph J, Chakrabarti M, Sharma S, Jayasudha R, Sama KC, Sontam B, Tyagi M, Narayanan R, Shivaji S (2019) New insights into culture negative endophthalmitis by unbiased next generation sequencing. Sci Rep 9(1):844. https://doi.org/10.1038/s41598-018-37502-w. PMID: 30696908; PMCID: PMC6351655.2416-4. PMID: 29515160; PMCID: PMC5841358

di Giovine FS, Duff GW (1990) Interleukin 1: the first interleukin. Immunol Today 11(1):13–20. https://doi.org/10.1016/0167-5699(90)90005-t. PMID: 2405873

Dick AD, Tugal-Tutkun I, Foster S, Zierhut M, Melissa Liew SH, Bezlyak V, Androudi S (2013) Secukinumab in the treatment of noninfectious uveitis: results of three randomized, controlled clinical trials. Ophthalmology 120(4):777–787. https://doi.org/10.1016/j.ophtha.2012.09.040. Epub 2013 Jan 3. PMID: 2329098

Dinarello CA (2000) Proinflammatory cytokines. Chest 118(2):503–508. https://doi.org/10.1378/chest.118.2.503. PMID: 10936147

Dinarello CA (2007) Historical insights into cytokines. Eur J Immunol 37(Suppl 1):S34–S45. https://doi.org/10.1002/eji.200737772

Dombi T, Kwok KK, Sultan MB (2012) A retrospective, pooled data analysis of the safety of pegaptanib sodium in the treatment of age-related macular degeneration in subjects with or

without diabetes mellitus. BMC Ophthalmol 12:37. https://doi.org/10.1186/1471-2415-12-37. PMID: 22871086; PMCID: PMC3472216
Domenici L, Origlia N, Falsini B et al (2014) Rescue of retinal function by BDNF in a mouse model of glaucoma. PLoS One 9:e115579. https://doi.org/10.1371/journal.pone.0115579
Donders FC (1855) v Graefes Arch Ophthalmol 1(2):106 (cited in Duke-Elder S, Dobree JH (eds.) (1967) System of ophthalmology, diseases of the retina, vol. X. Henry Kimpton, London, p 577)
Duke-Elder S, Dobree JH (eds) (1967) System of ophthalmology, diseases of the retina, vol X. Henry Kimpton, London, p 578
Duncan JL (2019) Ciliary neurotrophic factor treatment improves retinal structure and function in macular telangiectasia type 2. Ophthalmology 126:550–551
Eastlake K, Wang W, Jayaram H, Murray-Dunning C, Carr AJF, Ramsden CM, Vugler A, Gore K, Clemo N, Stewart M et al (2019) Phenotypic and functional characterization of Muller glia isolated from induced pluripotent stem cell-derived retinal organoids: improvement of retinal ganglion cell function upon transplantation. Stem Cells Transl Med 8(8):775–784. https://doi.org/10.1002/sctm.18-0263
Eastlake K, Luis J, Limb GA (2020) Potential of Müller cells for retinal neuroprotection. Curr Eye Res 45:339–348. https://doi.org/10.1080/02713683.2019.1648831
Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA (2005) Complement factor H polymorphism and age-related macular degeneration. Science 308(5720):421–424. https://doi.org/10.1126/science.1110189. Epub 2005 Mar 10. PMID: 15761121
Ellis S, Buchberger A, Holder J, Orhan E, Hughes J (2020) GT005, a gene therapy for the treatment of dry age-related macular degeneration (AMD). Invest Ophthalmol Vis Sci 61(7):2295
Eyetech Study Group (2002) Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina 22:143–152
Eyetech Study Group (2003) Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. Ophthalmology 110:979–986
Falese L, Sandza K, Yates B, Triffault S, Gangar S, Long B, Tsuruda L, Carter B, Vettermann C, Zoog SJ, Fong S (2017) Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther 24(12):768–778. https://doi.org/10.1038/gt.2017.95
Fenner TE, Garweg J, Hufert FT, Boehnke M, Schmitz H (1991) Diagnosis of human cytomegalovirus-induced retinitis in human immunodeficiency virus type 1-infected subjects by using the polymerase chain reaction. J Clin Microbiol 29(11):2621–2622. https://doi.org/10.1128/JCM.29.11.2621-2622.1991. PMID: 1663513; PMCID: PMC270387
Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25(4):581–611. https://doi.org/10.1210/er.2003-0027. PMID: 15294883
Ferrara N (2011) From the discovery of vascular endothelial growth factor to the introduction of avastin in clinical trials—an interview with Napoleone Ferrara by Domenico Ribatti. Int J Dev Biol 55(4-5):383–388. https://doi.org/10.1387/ijdb.103216dr. PMID: 21858763
Ferrara N (2016) VEGF and intraocular neovascularization: from discovery to therapy. Transl Vis Sci Technol 5(2):10. https://doi.org/10.1167/tvst.5.2.10. Published 2016 Mar 11
Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161(2):851–858. https://doi.org/10.1016/0006-291x(89)92678-8. PMID: 2735925
Ferreira VL, Borba HHL, Bonetti AF, Leonart LP, Pontarolo R (2019) Cytokines and interferons: types and functions. In: Autoantibodies and cytokines. https://doi.org/10.5772/intechopen.74550
Fontanarosa PB, DeAngelis CD (2002) Basic science and translational research in JAMA. JAMA 287(13):1728. https://doi.org/10.1001/jama.287.13.1728. PMID: 11926900
Fort DG, Herr TM, Shaw PL, Gutzman KE, Starren JB (2017) Mapping the evolving definitions of translational research. J Clin Transl Sci 1:60–66

Fox GM, Crouse CA, Chuang EL, Pflugfelder SC, Cleary TJ, Nelson SJ, Atherton SS (1991) Detection of herpesvirus DNA in vitreous and aqueous specimens by the polymerase chain reaction. Arch Ophthalmol 109(2):266–271. https://doi.org/10.1001/archopht.1991.01080020112054. PMID: 1847043

Frank RN, Amin RH, Eliott D, Puklin JE, Abrams GW (1996) Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. Am J Ophthalmol 122(3):393–403. https://doi.org/10.1016/s0002-9394(14)72066-5. PMID: 8794712

Fritsche LG, Loenhardt T, Janssen A, Fisher SA, Rivera A, Keilhauer CN, Weber BH (2008) Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 40(7):892–896. https://doi.org/10.1038/ng.170. Epub 2008 May 30. PMID: 18511946

Fukunaga H, Kaburaki T, Shirahama S, Tanaka R, Murata H, Sato T, Takeuchi M, Tozawa H, Urade Y, Katsura M, Kobayashi M, Wada Y, Soga H, Kawashima H, Kohro T, Aihara M (2020) Analysis of inflammatory mediators in the vitreous humor of eyes with pan-uveitis according to aetiological classification. Sci Rep 10(1):2783. https://doi.org/10.1038/s41598-020-59666-0. PMID: 32066796; PMCID: PMC7026072

Garafalo AV, Cideciyan AV, Héon E, Sheplock R, Pearson A, WeiYang YC, Sumaroka A, Aguirre GD, Jacobson SG (2020) Progress in treating inherited retinal diseases: early subretinal gene therapy clinical trials and candidates for future initiatives. Prog Retin Eye Res 77:100827. https://doi.org/10.1016/j.preteyeres.2019.100827. Epub 2019 Dec 30. PMID: 31899291

Gokoffski KK, Peng M, Alas B, Lam P (2020) Neuro-protection and neuro-regeneration of the optic nerve: recent advances and future directions. Curr Opin Neurol 33(1):93–105. https://doi.org/10.1097/WCO.0000000000000777. PMID: 31809331

Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, Gehrs K, Cramer K, Neel J, Bergeron J, Barile GR, Smith RT, AMD Genetics Clinical Study Group, Hageman GS, Dean M, Allikmets R (2006) Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 38(4):458–462. https://doi.org/10.1038/ng1750. Epub 2006 Mar 5. PMID: 16518403; PMCID: PMC2921703

Guimaraes TAC, Georgiou M, Bainbridge JWB, Michaelides M (2021) Gene therapy for neovascular age-related macular degeneration: rationale, clinical trials and future directions. Br J Ophthalmol 105(2):151–157. https://doi.org/10.1136/bjophthalmol-2020-316195. PMID: 32269060

Gupta A, Gupta V (2005) Tubercular posterior uveitis. Int Ophthalmol Clin 45(2):71–88. https://doi.org/10.1097/01.iio.0000155934.52589.e3. PMID: 15791159

Gupta V, Arora S, Gupta A, Ram J, Bambery P, Sehgal S (1998) Management of presumed intraocular tuberculosis: possible role of the polymerase chain reaction. Acta Ophthalmol Scand 76(6):679–682. https://doi.org/10.1034/j.1600-0420.1998.760609.x. PMID: 9881551

Gupta A, Gupta V, Arora S, Dogra MR, Bambery P (2001) PCR-positive tubercular retinal vasculitis: clinical characteristics and management. Retina 21(5):435–444. https://doi.org/10.1097/00006982-200110000-00004. PMID: 11642371

Gupta V, Gupta A, Arora S, Bambery P, Dogra MR, Agarwal A (2003) Presumed tubercular serpiginouslike choroiditis: clinical presentations and management. Ophthalmology 110(9):1744–1749. https://doi.org/10.1016/S0161-6420(03)00619-5. PMID: 13129872

Gupta V, Gupta A, Arora S, Sachdeva N, Bambery P (2005) Simultaneous choroidal tuberculoma and epididymo-orchitis caused by Mycobacterium tuberculosis. Am J Ophthalmol 140(2):310–312. https://doi.org/10.1016/j.ajo.2005.01.023. PMID: 16086954

Guymer C, Wood JP, Chidlow G, Casson RJ (2019) Neuroprotection in glaucoma: recent advances and clinical translation. Clin Exp Ophthalmol 47(1):88–105. https://doi.org/10.1111/ceo.13336. Epub 2018 Jul 1. PMID: 29900639

Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, Spencer KL, Kwan SY, Noureddine M, Gilbert JR, Schnetz-Boutaud N, Agarwal A, Postel EA, Pericak-Vance MA (2005) Complement factor H variant increases the risk of age-related macular degeneration.

Science 308(5720):419–421. https://doi.org/10.1126/science.1110359. Epub 2005 Mar 10. PMID: 1576112

Harper MM, Boese EA, Kardon RH, Ledolter J, Kuehn MH (2020) High correlation between glaucoma treatment with topical prostaglandin analogs and bdnf immunoreactivity in human retina. Curr Eye Res 27:1–7. https://doi.org/10.1080/02713683.2020.1822417. PMID: 32985274

Hasin Y, Seldin M, Lusis A (2017) Multi-omics approaches to disease. Genome Biol 18(1):83. https://doi.org/10.1186/s13059-017-1215-1. PMID: 28476144; PMCID: PMC5418815

Hassan M, Karkhur S, Bae JH, Halim MS, Ormaechea MS, Onghanseng N, Nguyen NV, Afridi R, Sepah YJ, Do DV, Nguyen QD (2019) New therapies in development for the management of non-infectious uveitis: a review. Clin Exp Ophthalmol 47(3):396–417. https://doi.org/10.1111/ceo.13511. PMID: 30938012

He S, Stankowska DL, Ellis DZ, Krishnamoorthy RR, Yorio T (2018) Targets of neuroprotection in glaucoma. J Ocul Pharmacol Ther 34(1–2):85–106. https://doi.org/10.1089/jop.2017.0041. Epub 2017 Aug 18. PMID: 28820649; PMCID: PMC5963639

Heier JS, Brown DM, Chong V, Korobelnik JF, Kaiser PK, Nguyen QD, Kirchhof B, Ho A, Ogura Y, Yancopoulos GD, Stahl N, Vitti R, Berliner AJ, Soo Y, Anderesi M, Groetzbach G, Sommerauer B, Sandbrink R, Simader C, Schmidt-Erfurth U, VIEW 1 and VIEW 2 Study Groups (2012) Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration. Ophthalmology 119(12):2537–2548. https://doi.org/10.1016/j.ophtha.2012.09.006. Epub 2012 Oct 17. Erratum in: Ophthalmology (2013) 120(1):209–210. PMID: 23084240

Heier JS, Korobelnik JF, Brown DM, Schmidt-Erfurth U, Do DV, Midena E, Boyer DS, Terasaki H, Kaiser PK, Marcus DM, Nguyen QD, Jaffe GJ, Slakter JS, Simader C, Soo Y, Schmelter T, Vitti R, Berliner AJ, Zeitz O, Metzig C, Holz FG (2016) Intravitreal aflibercept for diabetic macular edema: 148-week results from the VISTA and VIVID studies. Ophthalmology 123(11):2376–2385. https://doi.org/10.1016/j.ophtha.2016.07.032. Epub 2016 Sep 17. PMID: 27651226

Heier JS, Kherani S, Desai S, Dugel P, Kaushal S, Cheng SH, Delacono C, Purvis A, Richards S, Le-Halpere A, Connelly J, Wadsworth SC, Varona R, Buggage R, Scaria A, Campochiaro PA (2017) Intravitreous injection of AAV2-sFLT01 in patients with advanced neovascular age-related macular degeneration: a phase 1, open-label trial. Lancet 390(10089):50–61. https://doi.org/10.1016/S0140-6736(17)30979-0. Epub 2017 May 17. Erratum in: Lancet (2017) 390(10089):28. PMID: 28526489

von Helmholtz H (1851) https://en.wikipedia.org/wiki/Hermann_von_Helmholtz

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A 99(17):11393–11398. https://doi.org/10.1073/pnas.172398299. Epub 2002 Aug 12. PMID: 12177445; PMCID: PMC123267

Holz FG, Sadda SR, Busbee B, Chew EY, Mitchell P, Tufail A, Brittain C, Ferrara D, Gray S, Honigberg L, Martin J, Tong B, Ehrlich JS, Bressler NM (2018) Chroma and Spectri Study Investigators. Efficacy and safety of lampalizumab for geographic atrophy due to age-related macular degeneration: chroma and spectri phase 3 randomized clinical trials. JAMA Ophthalmol 136(6):666–677. https://doi.org/10.1001/jamaophthalmol.2018.1544. PMID: 29801123; PMCID: PMC6145777

Hornan D, Edmeades N, Krishnan R, Khan J, Lochhead J (2010) Use of pegaptanib for recurrent and non-clearing vitreous haemorrhage in proliferative diabetic retinopathy. Eye (Lond) 24(8):1315–1319. https://doi.org/10.1038/eye.2010.14. Epub 2010 Mar 12. PMID: 20224599

Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, Antoni C, Draelos Z, Gold MH, Psoriasis Study Group, Durez P, Tak PP, Gomez-Reino JJ, Rheumatoid Arthritis Study Group, Foster CS, Kim RY, Samson CM, Falk NS, Chu DS, Callanan D, Nguyen QD, Uveitis Study Group, Rose K, Haider A, Di Padova F (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. Sci Transl Med 2(52):52ra72. https://doi.org/10.1126/scitranslmed.3001107. PMID: 20926833

Isaacs A, Lindenmann J (1957) Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 147(927):258–267. https://doi.org/10.1098/rspb.1957.0048. PMID: 13465720

Isaacs A, Lindenmann J, Valentine RC (1957) Virus interference. II. Some properties of interferon. Proc R Soc Lond B Biol Sci 147(927):268–273. https://doi.org/10.1098/rspb.1957.0049. PMID: 13465721

Jaffe GJ, Westby K, Csaky KG, Monés J, Pearlman JA, Patel SS, Joondeph BC, Randolph J, Masonson H, Rezaei KA (2021) C5 Inhibitor avacincaptad pegol for geographic atrophy due to age-related macular degeneration: a randomized pivotal phase 2/3 trial. Ophthalmology 128(4): 576–586. https://doi.org/10.1016/j.ophtha.2020.08.027. S0161-6420(20)30845-9. PMID: 32882310

Jawad S, Liu B, Agron E, Nussenblatt RB, Sen HN (2013) Elevated serum levels of interleukin-17A in uveitis patients. Ocul Immunol Inflamm 21(6):434–439. https://doi.org/10.3109/09273948.2013.815786. Epub 2013 Aug 19. PMID: 23957503; PMCID: PMC5569243

Jayaram H, Jones MF, Eastlake K, Cottrill PB, Becker S, Wiseman J, Khaw PT, Limb GA (2014) Transplantation of photoreceptors derived from human Muller glia restore rod function in the P23H rat. Stem Cells Transl Med 3(3):323–333. https://doi.org/10.5966/sctm.2013-0112. Epub 2014 Jan 29. PMID: 24477073; PMCID: PMC3952927

Ji JZ, Elyaman W, Yip HK, Lee VW, Yick LW, Hugon J, So KF (2004) CNTF promotes survival of retinal ganglion cells after induction of ocular hypertension in rats: the possible involvement of STAT3 pathway. Eur J Neurosci 19(2):265–272. https://doi.org/10.1111/j.0953-816x.2003.03107.x. PMID: 14725620

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096): 816–821. https://doi.org/10.1126/science.1225829. Epub 2012 Jun 28. PMID: 22745249; PMCID: PMC6286148

Kahn P, Weiss M, Imundo LF, Levy DM (2006) Favorable response to high-dose infliximab for refractory childhood uveitis. Ophthalmology 113(5):860–4.e2. https://doi.org/10.1016/j.ophtha.2006.01.005. Epub 2006 Mar 20. PMID: 16545455

KalininaAyuso V, de Boer JH, Byers HL, Coulton GR, Dekkers J, de Visser L, van Loon AM, Schellekens PA, Rothova A, de Groot-Mijnes JD (2013) Intraocular biomarker identification in uveitis associated with juvenile idiopathic arthritis. Invest Ophthalmol Vis Sci 54(5): 3709–3720. https://doi.org/10.1167/iovs.12-10865. PMID: 23633652

Kamba T, McDonald DM (2007) Mechanisms of adverse effects of anti-VEGF therapy for cancer. Br J Cancer 96(12):1788–1795. https://doi.org/10.1038/sj.bjc.6603813. Epub 2007 May 22. PMID: 17519900; PMCID: PMC2359962

Kansara V, Muya L, Wan CR, Ciulla TA (2020) Suprachoroidal delivery of viral and nonviral gene therapy for retinal diseases. J Ocul Pharmacol Ther 36(6):384–392. https://doi.org/10.1089/jop.2019.0126. Epub 2020 Apr 7. PMID: 32255727; PMCID: PMC7404827

Karkhur S, Hasanreisoglu M, Vigil E, Halim MS, Hassan M, Plaza C, Nguyen NV, Afridi R, Tran AT, Do DV, Sepah YJ, Nguyen QD (2019) Interleukin-6 inhibition in the management of non-infectious uveitis and beyond. J Ophthalmic Inflamm Infect 9(1):17. https://doi.org/10.1186/s12348-019-0182-y

Kirstahler P, Bjerrum SS, Friis-Møller A, la Cour M, Aarestrup FM, Westh H, Pamp SJ (2018) Genomics-based identification of microorganisms in human ocular body fluid. Sci Rep 8(1): 4126. https://doi.org/10.1038/s41598-018-22416-4. PMID: 29515160; PMCID: PMC5841358

Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J (2005) Complement factor H polymorphism in age-related macular degeneration. Science 308(5720):385–389. https://doi.org/10.1126/science.1109557. Epub 2005 Mar 10. PMID: 15761122; PMCID: PMC1512523

Knox BE (2012) Translational science in ophthalmology. J Ophthalmic Vis Res 7(1):1. PMID: 22737378; PMCID: PMC3381096

Knox CM, Chandler D, Short GA, Margolis TP (1998) Polymerase chain reaction-based assays of vitreous samples for the diagnosis of viral retinitis. Use in diagnostic dilemmas. Ophthalmology

105(1):37–44; discussion 44–45. https://doi.org/10.1016/s0161-6420(98)71127-2. PMID: 9442777

Kobayashi T, Oku H, Fukuhara M, Kojima S, Komori A, Ichikawa M, Katsumura K, Kobayashi M, Sugiyama T, Ikeda T (2005) Endothelin-1 enhances glutamate-induced retinal cell death, possibly through ETA receptors. Invest Ophthalmol Vis Sci 46(12):4684–4690. https://doi.org/10.1167/iovs.05-0785. PMID: 16303965

Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, Li W, Connolly E, O'Neill CA, Miller JW (2002) Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 120(3):338–346. https://doi.org/10.1001/archopht.120.3.338. PMID: 11879138

Kuiper JJ, Mutis T, de Jager W, de Groot-Mijnes JD, Rothova A (2011) Intraocular interleukin-17 and proinflammatory cytokines in HLA-A29-associated birdshot chorioretinopathy. Am J Ophthalmol 152(2):177–182.e1. https://doi.org/10.1016/j.ajo.2011.01.031. Epub 2011 May 13. PMID: 21570674

Kulski JK (2016) Next-generation sequencing—an overview of the history, tools, and "omic" applications. Intech Open. https://books.google.co.in/books?id=l-WjzQEACAAJ. https://doi.org/10.5772/61964

Kumar A, Singh MP, Bansal R, Gupta A, Ram J, Ratho RK (2017) Development and evaluation of multiplex real-time PCR for diagnosis of HSV-1, VZV, CMV, and Toxoplasma gondii in patients with infectious uveitis. Diagn Microbiol Infect Dis 89(3):191–196. https://doi.org/10.1016/j.diagmicrobio.2017.08.002. Epub 2017 Aug 9. PMID: 28911798

Kvanta A, Algvere PV, Berglin L, Seregard S (1996) Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest Ophthalmol Vis Sci 37(9):1929–1934. PMID: 8759365

Letko E, Yeh S, Foster CS, Pleyer U, Brigell M, Grosskreutz CL, AIN457A2208 Study Group (2015) Efficacy and safety of intravenous secukinumab in noninfectious uveitis requiring steroid-sparing immunosuppressive therapy. Ophthalmology 122(5):939–948. https://doi.org/10.1016/j.ophtha.2014.12.033. Epub 2015 Jan 29. PMID: 25638011

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246(4935):1306–1309. https://doi.org/10.1126/science.2479986. PMID: 2479986

Liebreich R (1861) Ravin JG, Kenyon C. From von Graefe's clinic to the Ecole des Beaux-Arts. The meteoric career of Richard Liebreich. Surv Ophthalmol. 1992 Nov–Dec;37(3):221–8. https://doi.org/10.1016/0039-6257(92)90139-k. PMID: 1475755

Li Q, Miller R, Han PY, Pang J, Dinculescu A, Chiodo V, Hauswirth WW (2008) Intraocular route of AAV2 vector administration defines humoral immune response and therapeutic potential. Mol Vis 14:1760–1769. PMID: 18836574; PMCID: PMC25598

Li Z, Breitwieser FP, Lu J, Jun AS, Asnaghi L, Salzberg SL, Eberhart CG (2018) Identifying corneal infections in formalin-fixed specimens using next generation sequencing. Invest Ophthalmol Vis Sci 59(1):280–288. https://doi.org/10.1167/iovs.17-21617

Liao DS, Grossi FV, El Mehdi D, Gerber MR, Brown DM, Heier JS, Wykoff CC, Singerman LJ, Abraham P, Grassmann F, Nuernberg P, Weber BHF, Deschatelets P, Kim RY, Chung CY, Ribeiro RM, Hamdani M, Rosenfeld PJ, Boyer DS, Slakter JS, Francois CG (2020) Complement C3 inhibitor pegcetacoplan for geographic atrophy secondary to age-related macular degeneration: a randomized phase 2 trial. Ophthalmology 127(2):186–195. https://doi.org/10.1016/j.ophtha.2019.07.011. Epub 2019 Jul 16. PMID: 31474439

Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR (1996) Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 37(5):855–868. PMID: 8603870

Lopez-Gonzalez R, Loza E, Jover JA, Benitez Del Castillo JM, Mendez R, Hernandez-Garcia C, Pato E (2009) Treatment of refractory posterior uveitis with infliximab: a 7-year follow-up

study. Scand J Rheumatol 38(1):58–62. https://doi.org/10.1080/03009740802366076. PMID: 18991187

Ma L, Jakobiec FA, Dryja TP (2019) A review of next-generation sequencing (NGS): applications to the diagnosis of ocular infectious diseases. Semin Ophthalmol 34(4):223–231. https://doi.org/10.1080/08820538.2019.1620800. Epub 2019 Jun 6. PMID: 31170015

Maguire AM, Russell S, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, Marshall KA, McCague S, Reichert H, Davis M, Simonelli F, Leroy BP, Wright JF, High KA, Bennett J (2019) Efficacy, safety, and durability of voretigene neparvovec-rzyl in RPE65 mutation-associated inherited retinal dystrophy: results of phase 1 and 3 trials. Ophthalmology 126(9):1273–1285. https://doi.org/10.1016/j.ophtha.2019.06.017. Epub 2019 Jun 22. PMID: 31443789

Martin KR, Quigley HA, Zack DJ et al (2003) Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in a rat glaucoma model. Invest Ophthalmol Vis Sci 44(10):4357–4365. https://doi.org/10.1167/iovs.02-1332. PMID: 14507880

McCann JD, Margolis TP, Wong MG, Kuppermann BD, Luckie AP, Schwartz DM, Irvine AR, Ai E (1995) A sensitive and specific polymerase chain reaction-based assay for the diagnosis of cytomegalovirus retinitis. Am J Ophthalmol 120(2):219–226. https://doi.org/10.1016/s0002-9394(14)72610-8. PMID: 7639306

Mehta H, Kim LN, Mathis T, Zalmay P, Ghanchi F, Amoaku WM, Kodjikian L (2020) Trends in real-world neovascular AMD treatment outcomes in the UK. Clin Ophthalmol 14:3331–3342. https://doi.org/10.2147/OPTH.S275977. PMID: 33116384; PMCID: PMC756907

Michels S, Rosenfeld PJ (2004) Ranibizumab therapy for neovascular age-related macular degeneration. Retin Physician 1:16–22. https://www.retinalphysician.com/issues/2004/august-2004/ranibizumab-therapy-for-neovascular-age-related-ma

Michels S, Rosenfeld PJ, Puliafito CA, Marcus EN, Venkatraman AS (2005) Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration twelve-week results of an uncontrolled open-label clinical study. Ophthalmology 112(6):1035–1047. https://doi.org/10.1016/j.ophtha.2005.02.007. PMID: 15936441

Micklisch S, Lin Y, Jacob S et al (2017) Age-related macular degeneration associated polymorphism rs10490924 in *ARMS2* results in deficiency of a complement activator. J Neuroinflammation 14:4. https://doi.org/10.1186/s12974-016-0776-3

Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B et al (1994) Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 145(3):574–584. PMID: 7521577; PMCID: PMC1890317

Miserocchi E, Giuffrè C, Cornalba M, Pontikaki I, Cimaz R (2020) JAK inhibitors in refractory juvenile idiopathic arthritis-associated uveitis. Clin Rheumatol 39(3):847–851. https://doi.org/10.1007/s10067-019-04875-w. Epub 2020 Jan 2. PMID: 31897953

Montoya JG, Parmley S, Liesenfeld O, Jaffe GJ, Remington JS (1999) Use of the polymerase chain reaction for diagnosis of ocular toxoplasmosis. Ophthalmology 106(8):1554–1563. https://doi.org/10.1016/S0161-6420(99)90453-0. PMID: 10442904

Moura-Coelho N, Tavares Ferreira J, Bruxelas CP, Dutra-Medeiros M, Cunha JP, Pinto Proença R (2019) Rho kinase inhibitors-a review on the physiology and clinical use in Ophthalmology. Graefes Arch Clin Exp Ophthalmol 257(6):1101–1117. https://doi.org/10.1007/s00417-019-04283-5. Epub 2019 Mar 7. PMID: 30843105

Nakano S, Tomaru Y, Kubota T, Takase H, Mochizuki M, Shimizu N, Sugita S, Strip PCR Project Group (2020) Evaluation of a multiplex strip PCR test for infectious uveitis: a prospective multicenter study. Am J Ophthalmol 213:252–259. https://doi.org/10.1016/j.ajo.2019.10.031. Epub 2019 Nov 28. PMID: 31785234

Nguyen QD, Brown DM, Marcus DM, Boyer DS, Patel S, Feiner L, Gibson A, Sy J, Rundle AC, Hopkins JJ, Rubio RG, Ehrlich JS, RISE and RIDE Research Group (2012) Ranibizumab for diabetic macular edema: results from 2 phase III randomized trials: RISE and RIDE. Ophthalmology 119(4):789–801. https://doi.org/10.1016/j.ophtha.2011.12.039. Epub 2012 Feb 11. PMID: 22330964

Nishi M, Hanashiro R, Moil S et al (1992) Polymerase chain reaction for the detection of the Varicella-zoster genome in ocular samples from patients with acute retinal necrosis. Am J Ophthalmol 114:603–609

Okumura N, Kinoshita S, Koizumi N (2017) Application of rho kinase inhibitors for the treatment of corneal endothelial diseases. J Ophthalmol 2017:2646904. https://doi.org/10.1155/2017/2646904. Epub 2017 Jul 2. PMID: 28751979; PMCID: PMC5511675

Ooi KG, Galatowicz G, Calder VL, Lightman SL (2006) Cytokines and chemokines in uveitis: is there a correlation with clinical phenotype? Clin Med Res 4(4):294–309. https://doi.org/10.3121/cmr.4.4.294. PMID: 17210978; PMCID: PMC1764804

Orrock JE, Ilowite NT (2016) Canakinumab for the treatment of active systemic juvenile idiopathic arthritis. Expert Rev Clin Pharmacol 9(8):1015–1024. https://doi.org/10.1080/17512433.2016.1204910. Epub 2016 Jul 6. PMID: 27367267

Osborne A, Khatib TZ, Songra L, Barber AC, Hall K, Kong GYX, Widdowson PS, Martin KR (2018) Neuroprotection of retinal ganglion cells by a novel gene therapy construct that achieves sustained enhancement of brain-derived neurotrophic factor/tropomyosin—related kinase receptor-B signaling. Cell Death Dis 9(10):1007. https://doi.org/10.1038/s41419-018-1041-8

Owyang AM, Issafras H, Corbin J, Ahluwalia K, Larsen P, Pongo E, Handa M, Horwitz AH, Roell MK, Haak-Frendscho M, Masat L (2011) XOMA 052, a potent, high-affinity monoclonal antibody for the treatment of IL-1β-mediated diseases. MAbs 3(1):49–60. https://doi.org/10.4161/mabs.3.1.13989. Epub 2011 Jan 1. PMID: 21048425; PMCID: PMC3038011

Patz A (1984) Retinal neovascularisation: early contributions of Professor Michaelson and recent observations. Br J Ophthalmol 68(1):42–46. https://doi.org/10.1136/bjo.68.1.42. PMID: 6197084; PMCID: PMC1040236

Payne AJ, Kaja S, Sabates NR, Koulen P (2013) A case for neuroprotection in ophthalmology: developments in translational research. Mo Med 110(5):429–436

Pennesi ME, Schlecther CL (2020) The evolution of retinal gene therapy: from clinical trials to clinical practice. Ophthalmology 127(2):148–150. https://doi.org/10.1016/j.ophtha.2019.12.003. Erratum in: Ophthalmology (2020) 127(4):557. PMID: 31973830

Pieragostino D, D'Alessandro M, di Ioia M, Di Ilio C, Sacchetta P, Del Boccio P (2015) Unraveling the molecular repertoire of tears as a source of biomarkers: beyond ocular diseases. Proteomics Clin Appl 9(1-2):169–186. https://doi.org/10.1002/prca.201400084. Epub 2015 Jan 12. PMID: 25488355

Powner MB, Gillies MC, Tretiach M, Scott A, Guymer RH, Hageman GS, Fruttiger M (2010) Perifoveal Müller cell depletion in a case of macular telangiectasia type 2. Ophthalmology 117(12):2407–2416. https://doi.org/10.1016/j.ophtha.2010.04.001. Epub 2010 Aug 3. PMID: 20678804; PMCID: PMC2974049

Price MO, Mehta JS, Jurkunas UV, Price FW Jr (2020) Corneal endothelial dysfunction: evolving understanding and treatment options. Prog Retin Eye Res 22:100904. https://doi.org/10.1016/j.preteyeres.2020.100904. PMID: 32977001

Qiu TG (2019) Transplantation of human embryonic stem cell-derived retinal pigment epithelial cells (MA09-hRPE) in macular degeneration. npj Regen Med 4:19. https://doi.org/10.1038/s41536-019-0081-8

Rakoczy EP, Magno AL, Lai CM, Pierce CM, Degli-Esposti MA, Blumenkranz MS, Constable IJ (2019) Three-year follow-up of phase 1 and 2a rAAV.sFLT-1 subretinal gene therapy trials for exudative age-related macular degeneration. Am J Ophthalmol 204:113–123. https://doi.org/10.1016/j.ajo.2019.03.006. Epub 2019 Mar 13. PMID: 30878487

Reddy A, Muhammad F, Lee D (2018) Biological therapies that target inflammatory cytokines to treat uveitis. In: Conference proceedings, pp 1–33. https://doi.org/10.5772/INTECHOPEN.82252. CorpusID:86671167

Reddy A, Muhammad F, Lee DJ (2019) Biological therapies that target inflammatory cytokines to treat uveitis. In: Advances in the diagnosis and management of uveitis. https://doi.org/10.5772/intechopen.82252

Rhee KD, Nusinowitz S, Chao K, Yu F, Bok D, Yang XJ (2013) CNTF-mediated protection of photoreceptors requires initial activation of the cytokine receptor gp130 in Müller glial cells. Proc Natl Acad Sci U S A 110(47):E4520–E4529. https://doi.org/10.1073/pnas.1303604110. Epub 2013 Nov 4. PMID: 24191003; PMCID: PMC3839707

Rodrigues MM, Spaeth GL, Sivalingam E, Weinreb S (1976) Histopathology of 150 trabeculectomy specimens in glaucoma. Trans Ophthalmol Soc U K 96(2):245–255. PMID: 1070878

Rosenfeld PJ, Dugel PU, Holz FG, Heier JS, Pearlman JA, Novack RL, Csaky KG, Koester JM, Gregory JK, Kubota R (2018) Emixustat hydrochloride for geographic atrophy secondary to age-related macular degeneration: a randomized clinical trial. Ophthalmology 125(10): 1556–1567. https://doi.org/10.1016/j.ophtha.2018.03.059. Epub 2018 Apr 30. Erratum in: Ophthalmology (2019) 126(3):471–472. PMID: 29716784

Rozing MP, Durhuus JA, Krogh Nielsen M, Subhi Y, Kirkwood TB, Westendorp RG, Sørensen TL (2020) Age-related macular degeneration: a two-level model hypothesis. Prog Retin Eye Res 76: 100825. https://doi.org/10.1016/j.preteyeres.2019.100825. Epub 2019 Dec 30. PMID: 31899290

Rudwaleit M, Rosenbaum JT, Landewé R, Marzo-Ortega H, Sieper J, van der Heijde D, Davies O, Bartz H, Hoepken B, Nurminen T, Deodhar A (2016) Observed incidence of uveitis following certolizumab pegol treatment in patients with axial spondyloarthritis. Arthritis Care Res 68(6): 838–844. https://doi.org/10.1002/acr.22848

Ryan SJ, Hinton DR, Ogden TE, Rao NA (2002) Translational research in Ophthalmology. Arch Ophthalmol 120:389–390

Sacchetti M, Lambiase A, Schmidl D et al (2020) Effect of recombinant human nerve growth factor eye drops in patients with dry eye: a phase IIa, open label, multiple-dose study. Br J Ophthalmol 104(1):127–135. https://doi.org/10.1136/bjophthalmol-2018-312470

Sadiq MA, Hassan M, Afridi R et al (2020) Posterior segment inflammatory outcomes assessed using fluorescein angiography in the STOP-UVEITIS study. Int J Retin Vitr 6:47. https://doi.org/10.1186/s40942-020-00245-w

Sahni J, Patel SS, Dugel PU, Khanani AM, Jhaveri CD, Wykoff CC, Hershberger VS, Pauly-Evers M, Sadikhov S, Szczesny P, Schwab D, Nogoceke E, Osborne A, Weikert R, Fauser S (2019) Simultaneous inhibition of angiopoietin-2 and vascular endothelial growth factor-A with faricimab in diabetic macular edema: BOULEVARD phase 2 randomized trial. Ophthalmology 126(8):1155–1170. https://doi.org/10.1016/j.ophtha.2019.03.023. Epub 2019 Mar 21. PMID: 30905643

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74(12):5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman JP, Davis JL, Heilwell G, Spirn M, Maguire J, Gay R, Bateman J, Ostrick RM, Morris D, Vincent M, Anglade E, Del Priore LV, Lanza R (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385(9967):509–516. https://doi.org/10.1016/S0140-6736(14)61376-3. Epub 2014 Oct 15. PMID: 25458728

Schwartz SD, Tan G, Hosseini H, Nagiel A (2016) Subretinal transplantation of embryonic stem cell-derived retinal pigment epithelium for the treatment of macular degeneration: an assessment at 4 years. Invest Ophthalmol Vis Sci 57(5):ORSFc1–ORSFc9. https://doi.org/10.1167/iovs.15-18681. PMID: 27116660

Seddon JM, Francis PJ, George S, Schultz DW, Rosner B, Klein ML (2007) Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. JAMA 297(16):1793–1800. https://doi.org/10.1001/jama.297.16.1793. Erratum in: JAMA (2007) 297(23):2585. PMID: 17456821

Semba RD, Enghild JJ, Venkatraman V, Dyrlund TF, Van Eyk JE (2013) The Human Eye Proteome Project: perspectives on an emerging proteome. Proteomics 13(16):2500–2511. https://doi.org/10.1002/pmic.201300075

Sepah YJ, Velez G, Tang PH, Yang J, Chemudupati T, Li AS, Nguyen QD, Bassuk AG, Mahajan VB (2020) Proteomic analysis of intermediate uveitis suggests myeloid cell recruitment and implicates IL-23 as a therapeutic target. Am J Ophthalmol Case Rep 18:100646. https://doi.org/10.1016/j.ajoc.2020.100646. PMID: 32274442; PMCID: PMC7132169

Sharma K, Gupta V, Bansal R, Sharma A, Sharma M, Gupta A (2013) Novel multi-targeted polymerase chain reaction for diagnosis of presumed tubercular uveitis. J Ophthalmic Inflamm Infect 3(1):25. https://doi.org/10.1186/1869-5760-3-25. Published 2013 Jan 28

Sharma K, Sharma A, Bansal R, Fiorella PD, Gupta A (2014) Drug-resistant tubercular uveitis. J Clin Microbiol 52(11):4113–4114. https://doi.org/10.1128/JCM.01918-14. Epub 2014 Sep 3. PMID: 25187635; PMCID: PMC4313252

Sharma K, Bansal R, Sharma A, Gupta A, Fiorella PD (2015) Successful treatment of rifampicin-resistant intraocular tuberculosis. Ocul Immunol Inflamm 23(1):93–96. https://doi.org/10.3109/09273948.2014.888084. Epub 2014 Mar 21. PMID: 24654625

Sharma K, Gupta A, Sharma M, Sharma A, Bansal R, Sharma SP, Singh RD, Gupta V (2019) The emerging challenge of diagnosing drug-resistant tubercular uveitis: experience of 110 eyes from North India. Ocul Immunol Inflamm 3:1–8. https://doi.org/10.1080/09273948.2019.1655581. PMID: 31580170

Shen J, Kim J, Tzeng SY, Ding K, Hafiz Z, Long D, Wang J, Green JJ, Campochiaro PA (2020) Suprachoroidal gene transfer with nonviral nanoparticles. Sci Adv 6(27):eaba1606. https://doi.org/10.1126/sciadv.aba1606. PMID: 32937452; PMCID: PMC7458446

Sheppard J, Joshi A, Betts KA, Hudgens S, Tari S, Chen N, Skup M, Dick AD (2017) Effect of adalimumab on visual functioning in patients with noninfectious intermediate uveitis, posterior uveitis, and panuveitis in the VISUAL-1 and VISUAL-2 trials. JAMA Ophthalmol 135(6):511–518. https://doi.org/10.1001/jamaophthalmol.2017.0603. PMID: 28426849; PMCID: PMC5847080

Shirinsky IV, Biryukova AA, Kalinovskaya NY, Shirinsky VS (2020) Tear cytokines as potential biomarkers in non-infectious uveitis: posthoc analysis of a randomised clinical trial. Graefes Arch Clin Exp Ophthalmol 258:1813–1819. https://doi.org/10.1007/s00417-020-04707-7

Short GA, Margolis TP, Kuppermann BD, Irvine AR, Martin DF, Chandler D (1997) A polymerase chain reaction-based assay for diagnosing varicella-zoster virus retinitis in patients with acquired immunodeficiency syndrome. Am J Ophthalmol 123(2):157–164. https://doi.org/10.1016/s0002-9394(14)71031-1. PMID: 9186120

Sieving PA, Caruso RC, Tao W, Coleman HR, Thompson DJ, Fullmer KR, Bush RA (2006) Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. Proc Natl Acad Sci U S A 103(10):3896–3901. https://doi.org/10.1073/pnas.0600236103. Epub 2006 Feb 27. PMID: 16505355; PMCID: PMC1383495

Singh R, Toor P, Parchand S, Sharma K, Gupta V, Gupta A (2012) Quantitative polymerase chain reaction for Mycobacterium tuberculosis in so-called Eales' disease. Ocul Immunol Inflamm 20(3):153–157. https://doi.org/10.3109/09273948.2012.658134. Epub 2012 Apr 9. PMID: 22486260

Suhler EB, Smith JR, Wertheim MS, Lauer AK, Kurz DE, Pickard TD, Rosenbaum JT (2005) A prospective trial of infliximab therapy for refractory uveitis: preliminary safety and efficacy outcomes. Arch Ophthalmol 123(7):903–912. https://doi.org/10.1001/archopht.123.7.903. PMID: 16009830

Sultan MB, Zhou D, Loftus J, Dombi T, Ice KS, Macugen 1013 Study Group (2011) A phase 2/3, multicenter, randomized, double-masked, 2-year trial of pegaptanib sodium for the treatment of diabetic macular edema. Ophthalmology 118(6):1107–1118. https://doi.org/10.1016/j.ophtha.2011.02.045. Epub 2011 May 6. PMID: 21529957

Sun JK, Wang PW, Taylor S, Haskova Z (2019) Durability of diabetic retinopathy improvement with As-needed ranibizumab: open-label extension of ride and rise studies. Ophthalmology 126(5):712–720. https://doi.org/10.1016/j.ophtha.2018.10.041. Epub 2018 Nov 9. PMID: 30419298

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676. https://doi.org/10.1016/j.cell.2006.07.024. Epub 2006 Aug 10. PMID: 16904174

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872. https://doi.org/10.1016/j.cell.2007.11.019. PMID: 18035408

Tanihara H, Inoue T, Yamamoto T, Kuwayama Y, Abe H, Araie M, K-115 Clinical Study Group (2013) Phase 2 randomized clinical study of a Rho kinase inhibitor, K-115, in primary open-angle glaucoma and ocular hypertension. Am J Ophthalmol 156(4):731–736. https://doi.org/10.1016/j.ajo.2013.05.016. Epub 2013 Jul 4. PMID: 23831221

Tanihara H, Inoue T, Yamamoto T, Kuwayama Y, Abe H, Suganami H, Araie M, K-115 Clinical Study Group (2015) Additive intraocular pressure-lowering effects of the rho kinase inhibitor ripasudil (K-115) combined with timolol or latanoprost: a report of 2 randomized clinical trials. JAMA Ophthalmol 133(7):755–761. https://doi.org/10.1001/jamaophthalmol.2015.0525. PMID: 25880207

Tanna AP, Johnson M (2018) Rho kinase inhibitors as a novel treatment for glaucoma and ocular hypertension. Ophthalmology 125(11):1741–1756. https://doi.org/10.1016/j.ophtha.2018.04.040. Epub 2018 Jul 12. PMID: 30007591; PMCID: PMC6188806

The Lasker/IRRF Initiative for Innovation in Vision Science (2014) Chapter 2: Restoring vision to the blind: optogenetics. Trans Vis Sci Tech 3(7):14–22. https://doi.org/10.1167/tvst.3.7.4

Therese KL, Jayanthi U, Madhavan HN (2005) Application of nested polymerase chain reaction (nPCR) using MPB 64 gene primers to detect Mycobacterium tuberculosis DNA in clinical specimens from extrapulmonary tuberculosis patients. Indian J Med Res 122(2):165–170. PMID: 16177475

Tolentino MJ, McLeod DS, Taomoto M, Otsuji T, Adamis AP, Lutty GA (2002) Pathologic features of vascular endothelial growth factor-induced retinopathy in the nonhuman primate. Am J Ophthalmol 133(3):373–385. https://doi.org/10.1016/s0002-9394(01)01381-2. PMID: 1186097

Touchard E, Benard R, Bigot K, Laffitte JD, Buggage R, Bordet T, Behar-Cohen F (2018) Non-viral ocular gene therapy, pEYS606, for the treatment of non-infectious uveitis: preclinical evaluation of the medicinal product. J Control Release 285:244–251. https://doi.org/10.1016/j.jconrel.2018.07.013. Epub 2018 Aug 1. PMID: 30009894

Tsai JC (2013) Canadian Journal of Ophthalmology Lecture: translational research advances in glaucoma neuroprotection. Can J Ophthalmol 48(3):141–145. https://doi.org/10.1016/j.jcjo.2013.02.003. PMID: 23769773

Tugal-Tutkun I, Pavesio C, De Cordoue A, Bernard-Poenaru O, Gül A (2018) Use of gevokizumab in patients with Behçet's disease uveitis: an international, randomized, double-masked, placebo-controlled study and open-label extension study. Ocul Immunol Inflamm 26(7):1023–1033. https://doi.org/10.1080/09273948.2017.1421233. Epub 2018 Jan 25. PMID: 29370572

Vallet H, Seve P, Biard L, Baptiste Fraison J, Bielefeld P, Perard L, Bienvenu B, Abad S, Rigolet A, Deroux A, Sene D, Perlat A, Marie I, Feurer E, Hachulla E, Fain O, Clavel G, Riviere S, Bouche PA, Gueudry J, Pugnet G, Le Hoang P, RescheRigon M, Cacoub P, Bodaghi B, Saadoun D, French Uveitis Network (2016) Infliximab versus adalimumab in the treatment of refractory inflammatory uveitis: a multicenter study from the French Uveitis Network. Arthritis Rheumatol 68(6):1522–1530. https://doi.org/10.1002/art.39667. PMID: 27015607

Varkey JB, Shantha JG, Crozier I, Kraft CS, Lyon GM, Mehta AK, Kumar G, Smith JR, Kainulainen MH, Whitmer S, Ströher U, Uyeki TM, Ribner BS, Yeh S (2015) Persistence of ebola virus in ocular fluid during convalescence. N Engl J Med 372(25):2423–2427. https://doi.org/10.1056/NEJMoa1500306. Epub 2015 May 7. Erratum in: N Engl J Med (2015) 372(25):2469. PMID: 25950269; PMCID: PMC4547451

Velez G, Mahajan VB (2020) Molecular surgery: proteomics of a rare genetic disease gives insight into common causes of blindness. iScience 23(11):101667. https://doi.org/10.1016/j.isci.2020.101667

Velez G, Roybal CN, Colgan D, Tsang SH, Bassuk AG, Mahajan VB (2016) Precision medicine: personalized proteomics for the diagnosis and treatment of idiopathic inflammatory disease.

JAMA Ophthalmol 134(4):444–448. https://doi.org/10.1001/jamaophthalmol.2015.5934. PMID: 26848019; PMCID: PMC4833518

Velez G, Bassuk AG, Colgan D, Tsang SH, Mahajan VB (2017) Therapeutic drug repositioning using personalized proteomics of liquid biopsies. JCI Insight 2(24):e97818. https://doi.org/10.1172/jci.insight.97818. PMID: 29263305; PMCID: PMC5752263

von Graefe A (1858) v Graefes Arch Ophthalmol 4(2):250 (cited in Duke-Elder S, Dobree JH (eds.) (1967) System of ophthalmology, diseases of the retina, vol. X. Henry Kimpton, London, p 578)

Wang Q, Zhao HS, Li L (2016) Association between complement factor I gene polymorphisms and the risk of age-related macular degeneration: a Meta-analysis of literature. Int J Ophthalmol 9(2):298–305. https://doi.org/10.18240/ijo.2016.02.23. PMID: 26949655; PMCID: PMC4761747

Woolf SH (2008) The meaning of translational research and why it matters. JAMA 299(2):211–213. https://doi.org/10.1001/jama.2007.26. PMID: 18182604

Writing Committee for the Diabetic Retinopathy Clinical Research Network, Gross JG, Glassman AR, Jampol LM, Inusah S, Aiello LP, Antoszyk AN, Baker CW, Berger BB, Bressler NM, Browning D, Elman MJ, Ferris FL 3rd, Friedman SM, Marcus DM, Melia M, Stockdale CR, Sun JK, Beck RW (2015) Panretinal photocoagulation vs intravitreous ranibizumab for proliferative diabetic retinopathy: a randomized clinical trial. JAMA 314(20):2137–2146. https://doi.org/10.1001/jama.2015.15217

Wu J, Bell OH, Copland DA, Young A, Pooley JR, Maswood R, Evans RS, Khaw PT, Ali RR, Dick AD, Chu CJ (2020) Gene therapy for glaucoma by ciliary body aquaporin 1 disruption using CRISPR-Cas9. Mol Ther 28(3):820–829. https://doi.org/10.1016/j.ymthe.2019.12.012. Epub 2020 Jan 10. PMID: 31981492; PMCID: PMC7054720

Yehoshua Z, de Amorim Garcia Filho CA, Nunes RP, Gregori G, Penha FM, Moshfeghi AA, Zhang K, Sadda S, Feuer W, Rosenfeld PJ (2014) Systemic complement inhibition with eculizumab for geographic atrophy in age-related macular degeneration: the COMPLETE study. Ophthalmology 121(3):693–701. https://doi.org/10.1016/j.ophtha.2013.09.044. Epub 2013 Nov 26. PMID: 24289920; PMCID: PMC4015213

Zarbin M (2020) What constitutes translational research? Implications for the scope of translational vision science and technology. Transl Vis Sci Technol 9(8):22. https://doi.org/10.1167/tvst.9.8.22. PMID: 32855869; PMCID: PMC7422760

Zhang D, Vetrivel L, Verkman AS (2002) Aquaporin deletion in mice reduces intraocular pressure and aqueous fluid production. J Gen Physiol 119(6):561–569. https://doi.org/10.1085/jgp.20028597. PMID: 12034763; PMCID: PMC2233864

Zhang Y, Kontos CD, Annex BH, Popel AS (2019) Angiopoietin-tie signaling pathway in endothelial cells: a computational model. iScience 20:497–511. https://doi.org/10.1016/j.isci.2019.10.006. Epub 2019 Oct 3. PMID: 31655061; PMCID: PMC6806670

Zhao JJ, Afshari NA (2016) Generation of human corneal endothelial cells via in vitro ocular lineage restriction of pluripotent stem cells. Invest Ophthalmol Vis Sci 57(15):6878–6884. https://doi.org/10.1167/iovs.16-20024. PMID: 28002562; PMCID: PMC5215466

Zhong X, Gutierrez C, Xue T et al (2014) Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun 5:4047. PubMed: 24915161

Zhu W, Gramlich OW, Laboissonniere L, Jain A, Sheffield VC, Trimarchi JM, Tucker BA, Kuehn MH (2016) Transplantation of iPSC-derived TM cells rescues glaucoma phenotypes in vivo. Proc Natl Acad Sci U S A 113(25):E3492–E3500. https://doi.org/10.1073/pnas.1604153113. Epub 2016 Jun 6. PMID: 27274060; PMCID: PMC4922164

Zhu W, Godwin CR, Cheng L, Scheetz TE, Kuehn MH (2020a) Transplantation of iPSC-TM stimulates division of trabecular meshwork cells in human eyes. Sci Rep 10(1):2905. https://doi.org/10.1038/s41598-020-59941-0. PMID: 32076077; PMCID: PMC7031365

Zhu D, Xie M, Gademann F et al (2020b) Protective effects of human iPS-derived retinal pigmented epithelial cells on retinal degenerative disease. Stem Cell Res Ther 11:98. https://doi.org/10.1186/s13287-020-01608-8

Rapid Eye Movement Sleep and Dream Sleep

6

Birendra Nath Mallick

Abstract

Consciousness is an attribute of the brain, by the brain, and for the brain. Our understanding of neurophysiological mechanism of consciousness is rudimentary. The experimental scientists describe three states of consciousness as wakefulness, non-rapid eye movement sleep (NREMS), and rapid eye movement sleep (REMS); most dreams appear intermittently during REMS. As the dream (REMS) may apparently be compared with wake-like condition within sleep (NREMS), I proposed that understanding of this state would provide a better handle to explore the neuronal basis of consciousness. These states are reversible and contiguous and, while seamlessly transiting from one to the other state in health and diseased conditions, may overlap to various extent. In this chapter, I have combined some neurophysiological evidence with philosophical wisdom proposed by the philosophers and sages of ancient India in the *Upanishads*. In my view, an all-inclusive, background state, the *T-state* may explain the neurophysiological basis of consciousness and its proportional masking and unmasking are expressions of classical states.

Keywords

Cognitive states · Dream · EEG · Sleep · *Thuriya* · *T-state* · Upanishadic view · Wakefulness

B. N. Mallick (✉)
Amity Institute of Neuropsychology and Neurosciences, Amity University, Noida, Uttar Pradesh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_6

6.1 Rapid Eye Movement Sleep and Dream Sleep

Our healthy existence depends on how best we (our body) negotiate with our environment, living or non-living. By and large, such negotiations are dependent on our knowledge of understanding about the self and the surrounding. The acquisition of knowledge depends on how best we can extract the information due to interaction of self and surrounding. Our knowledge and understanding of this universe are done by the brain and ultimately depend on its (brain's) conscious state. Historically, in the absence of better defining characteristics, based on physical movement, the consciousness has been divided into wakefulness and sleep states, the latter has been further divided into sleep and dream states. The dream state is unique, involuntary state that behaviorally although one is deep asleep, as if consciously one appears to experience wakefulness-like condition, interestingly without any volitional control. Classically, the modern science grossly classifies consciousness into three states: wakefulness, sleep, and dream. Notwithstanding, in the ancient literature, the *Upanishads* (Mandukya Upanishad), based on their then contemporary wisdom, the philosophers and sages of ancient India have categorized the consciousness into four states and termed them as *Jagriti* (waking), S*ushupti* (sleep), *Swapna* (dream), and *Thuriya*. It is high time that the modern science should take note of these states and possibly using the modern sensitive gadgets try finding a meeting point while explaining the ancient philosophical and conceptual states. In attempting so, this author has explained the *Thuriya* state (and termed it as *T-state*) as all-pervasive background basal substrate state on which the rest of the three states get expressed (Mallick and Mukhopadhyay 2011). It has been proposed that inputs to the brain, globally or locally, reversibly and proportionately suppress (masking and unmasking) such background *T-state* causing expression of classical states in health and diseases. The modern experimental science does not recognize or conceptualize such basal background state on which the classical three states and their variations get expressed. In the absence of consideration of such state, the missing link of easy seamless transition and switching among various states and gradation within the same state during health and diseases would continue to remain a mystery.

Since our existence, historically, based on one's personal experience, the humans were aware of the three states, viz., awake, sleep, and dream. Although behaviorally one could grossly define awake and sleep states by the presence or absence of physical activity, inactivity does not necessarily define always either non-awake or asleep. Also, one finds it difficult to differentiate between rest, quiet-awake, awake but rest, asleep, and so on. Although behavioral studies on sleep and waking were conducted, inherently, it has limitations such as possible subjective biasness. As one would not be able to objectively define wakefulness and sleep states, critical scientific and experimental studies were limited until about the first quarter of the twentieth century. Thereafter, as recording of the electrical activities from the brain, the electroencephalogram (EEG), was possible, the sleep and waking states could be objectively defined and identified (Berger 1929; Rechtschaffen and Kales 1968); however, still one did not have any clue of objective identification of dream state. Subsequently, additional recording of eye movement and electrical activity from the

neck muscles, the electro-oculogram (EOG) and electromyogram (EMG), respectively, enabled us deciphering not only gradation of wakefulness and sleep but the dream state was also identified. A series of studies through the second-third of the twentieth century using those electrophysiological recordings not only had put the death nail on the passive theory of sleep but it gave birth to the concept that not only awake but also sleep is an active phenomenon [*reviewed in detail in* Moruzzi 1972]. The electrophysiological recordings of EEG, EOG, and EMG showed in animals as well as in humans that intermittently during sleep a paradoxical state appears when the brain becomes more active (apparently comparable to awake) and the eyes show significant activity/movement (increased EOG) while the antigravity muscles show almost no activity (atonia in the EMG). Therefore, this state was termed as rapid eye movement sleep (REMS) (Aserinsky and Kleitman 1953; Aserinsky 1999; Jouvet 1999) or paradoxical sleep (Jouvet and Michel 1958; Jouvet 1999). Subsequently, it was observed that usually this state is associated with vivid dreams. Hence, it has been termed as dream sleep as well, largely by the psychologists (Dement 1960); however, the term REMS has been most widely used and will be used in this piece. The vivid dreams appear intermittently during this state when otherwise one is fast asleep and one does not have a voluntary control over this state, while apparently, it provides intermittent manifestation of waking-like mental activity within the sleeping state (dreaming). Therefore, ***I thought a thorough exploration and understanding of neuronal regulation of this REMS state might offer us an objective as well as a better handle to experimentally study the neuronal basis as well as regulation of conscious states***.

6.2 Brainstem, REM-OFF, and REM-ON Neurons

Discovery of REMS as defined by the electrophysiological criteria (EEG, EOG, and EMG) in the mid-twentieth century invited leading researchers of the time to relook and reinterpret previous studies as well as explore further the brain regions and specific neurons responsible for its control (Aserinsky and Kleitman 1953; Moruzzi 1972; Jouvet 1999; Mallick et al. 2011). Experiments using localized lesion and transection of brain regions concluded that neurons at the core of the brainstem play a critical role in REMS regulation, which however are modulated by many other brain regions (Siegel 1989; Mallick and Inoue 1999; Steriade and McCarley 2005). The brainstem possesses, among many other types of neurons, the locus coeruleus (LC) as the primary source of noradrenaline (NA)-ergic neurons in the brain and somewhat extended more laterally placed laterodorsal and pedunculopontine tegmentum (LDT/PPT) possessing predominantly acetylcholine (ACh)-ergic neurons. Subsequent studies have shown that the LC and LDT/PPT possess GABA-ergic and glutamatergic neurons and terminals as well. The PPT ACh-ergic neurons are mostly active during REMS only or significantly increase activity during REMS and have been termed as REM-ON neurons (McCarley and Hobson 1971). In contrast, the LC-NA-ergic neurons behave in an opposite manner and are active during wake as well as non-REMS (NREMS) and become almost silent during REMS; they have

been termed as REM-OFF neurons (Hobson et al. 1975, 1983; Aston-Jones and Bloom 1981). Based on these independent, isolated studies and following the Lotka-Volterra model, it was proposed that the REM-ON and REM-OFF neurons reciprocally inhibit each other and form the basic scaffold for the regulation of REMS (McCarley and Hobson 1975; Sakai 1988); however, detailed mechanism was unknown.

6.3 Cessation of LC-REM-OFF Neuronal Activity Is a Prerequisite for REMS Generation

In previous studies the REM-OFF and REM-ON neuronal activities were recorded independently, in isolated experiments and in separate animals. For confirmation, ideally it was preferred to record them simultaneously in real time and in the same animal during normal behavioral sleep-waking-REMS as well as during REMS deprivation (REMSD). To my mind, it was necessary to understand if the cessation of REM-OFF neurons is a prerequisite or a coincidental occurrence associated with REMS. For that, we needed to know the mechanism of inhibition of the REM-OFF neurons, the execution of triggering of REM-ON neurons, and finally, the temporal relationship between the reciprocal activities between those two types of neurons. The reciprocal relationship between those two types of neurons was supported by their behavior during 96 h of continuous REMSD in freely moving normally behaving animals (Mallick et al. 1990). Incidentally, this is the only documented report where the effect of REMSD was studied on the activities of REM-ON and REM-OFF neurons although in separate cats.

In a series of studies, first we hypothesized that if cessation of REM-OFF neuronal activities was a necessity for the generation of REMS, continued activation of those REM-OFF neurons should prevent appearance of REMS. In behaving chronically prepared rats, we showed that if the LC neurons were kept continuously active for a long time, REMS was indeed prevented (Alam et al. 1993; Singh and Mallick 1996). To the best of my knowledge, this is the first documented study (in the literature) in behaving animals to chronically activate specific deep region of the brain with very small electrical current for a reasonably long duration to simulate an involuntary instinct behavior. Subsequently, we recorded electrophysiological sleep-wakefulness-REMS and the REM-ON and REM-OFF neuronal activities simultaneously in the same chronically prepared behaving animals and showed that temporally indeed they showed reciprocal relationship in their activities not only during spontaneous expression of REMS but also during brainstem reticular formation activation-induced waking (Mallick et al. 1998). Possibly this is still the only study where REM-ON and REM-OFF neurons have been recorded simultaneously (together) in the same animal, at the same time, during change of spontaneous conscious states as well as upon experimentally induced change of behavioral state. Consolidation of findings of these studies validated the reciprocal relationship between the REM-OFF and the REM-ON neurons. However, the mechanism of

activation and cessation of REM-ON and REM-OFF neurons in relation with REMS was not known.

6.4 Normally Why REMS Appears Only After a Period of NREMS and Does Not Appear During Waking

Although REMS is an instinct behavior, normally it appears only after a period of NREMS has been expressed. Also, it is not expressed during waking, apparently comparable expression e.g., hallucination has been reported during waking and during REMS-behavior disorder (Mahowald et al. 1998; Manni et al. 2002; Arnulf 2013). We proposed that the waking- and NREMS-inducing brain areas should have opposite effects on the REM-ON and REM-OFF neurons. Indeed, it was observed in chronically prepared behaving cats that the waking- and NREMS-inducing brain areas have inhibitory and excitatory effects, respectively, on the REM-ON and REM-OFF neurons (Thankachan et al. 2001). The stimulation of the NREMS area, although did not significantly affect the REM-OFF neurons, excited the REM-ON neurons (Mallick et al. 2004). These findings offered neuronal (cellular) mechanism of regulation of REMS; most importantly, they suggested that the REM-OFF neurons, which were active, must cease activity, while REM-ON neurons increase activity for the generation of REMS. The next issue for confirmation was to explore their neurochemical regulation that modulates REMS in health and diseases.

6.5 The Role of GABA on the Inhibition and Disinhibition of REM-OFF and REM-ON Neurons for the Regulation of REMS

We have seen above that ACh-ergic REM-ON and NA-ergic REM-OFF neurons are likely to be reciprocally connected, while the LC-REM-OFF neurons must cease activity for the generation and maintenance of REMS. In the late 1980s, it was a challenge to investigate the neurochemical basis of inhibition of those LC-REM-OFF neurons for the generation of REMS particularly in behaving animals. Notwithstanding, in a related in vitro slice preparation study, it was shown that ACh-ergic agonist, carbachol, depolarized the LC neurons suggesting that ACh directly would not inhibit the NA-ergic neurons (Egan and North 1985).

We proposed that ACh-ergic REM-ON projections would stimulate the GABA-ergic interneurons, which then would inhibit the NA-ergic REM-OFF neurons in the LC. Our contention was supported by the then reports that the LC possesses ACh-ergic projections as well as GABA-interneurons. The challenge to explore how ACh stimulated the GABA-ergic neurons in the LC was further compounded by the fact that the investigation needed to be conducted in chronically prepared behaving animals and the small size of the LC in the rats. We designed ***neuro-micro-anatomo-pharmaco-physio-behavioral study*** using a combination of serial sequential multiple microinjections of 200 nL of agonist and antagonist into the LC of

surgically prepared chronic rats. Microinjection of such extra-small quantity of chemical into the brain of freely moving living animals for behavioral study was among the early studies and was used for the first time from this lab in the 1990s. ACh-ergic and GABA-ergic agonist and antagonist individually or in a sequence were bilaterally microinjected locally deep into the LC in chronically prepared normally behaving freely moving animals and electrophysiological waking-sleep-REMS recorded continuously for long hours. The findings indeed confirmed that ACh-ergic inputs into the LC acted on the GABA-ergic neurons and initiated REMS, possibly by inhibiting the REM-OFF neurons and thus, supported our contention (Alam et al. 1993; Kaur et al. 1997; Mallick et al. 2001).

Combining findings of others along with that of ours, we proposed that activation of the REM-ON neurons released ACh, which stimulated the GABA-ergic neurons in the LC and the released GABA inhibited the REM-OFF neurons resulting in the generation and maintenance of REMS. However, it was not known how the REM-ON neurons remain inhibited and subsequently become active to initiate REMS. Earlier we have shown that the REM-OFF neurons receive excitatory and inhibitory inputs from the wake- and NREMS-active areas in the brainstem, while the REM-ON neurons receive opposite influence (Mallick et al. 2004). Integrating findings from other researchers (Williams and Reiner 1993; Inglis and Winn 1995) as well as that of ours (Pal and Mallick 2004, 2006), it was proposed that the NA from the REM-OFF neurons inhibits the REM-ON neurons preventing the appearance of REMS during waking. Subsequently, when some yet unidentified conditions are satisfied, GABA-ergic inputs from the substantia nigra (SN) act presynaptically on the NA-ergic inputs (terminals) coming from the LC REM-OFF neurons onto the PPT REM-ON neurons to withdraw the NA-ergic inhibition from the ACh-ergic REM-ON neurons and trigger them initiating REMS (Pal and Mallick 2009; Mallick et al. 2012).

6.6 Mathematical Modeling and Computational Simulation Studies (In Silico)

The REMS is a unique, instinct state, which under normal condition does not appear during waking but gets expressed only after the appearance of NREMS for some duration. Independently we have shown how waking and NREMS regulating areas in the brain modulate the REM-OFF and REM-ON neurons and their neuropharmacology for the generation of REMS. However, the facts are that under normal condition every NREMS is not followed by REMS, varied duration of NREMS appears before the expression of REMS, all REMS episodes do not continue for the same duration, and REMS may end up in NREMS or waking while, under abnormal conditions, REMS-like state (hallucination) appears during waking. Therefore, it was likely to be that there is complex interaction among various neural networks maintaining waking and NREMS for the regulation of REMS. The complexity gets further compounded by the fact that the synaptic strengths of the component neural networks and that of the networks at large are dynamic. As it is almost impossible to

modulate one or more of these networks simultaneously in vivo in behaving animals under controlled conditions, we reconstructed the neuronal connections as obtained from the animal studies (described above) into a mathematical model. In the said mathematical model, the input and output strengths of the networks could be modified individually or in combination. Such study revealed that as long as the inhibitory input from the brainstem waking area onto the REM-ON neurons remains active, despite the inputs from SN, REMS (as defined by the activity of the REM-ON neurons) does not appear (Kumar et al. 2012). The limitation of the said mathematical modeling simulation study is that it does not allow behavioral study; however, it provides in-depth understanding at the neuronal network level, which is not possible by the behavioral study; thus, they are complimentary. This finding is significant as it provides experimental evidence why REMS does not appear during waking and suggests possible mechanism of appearance of dream-like state (hallucination) during waking in diseased condition. The importance and power of this model is that it may be used to explore the possible neurophysiological and neuropharmacological deviations in association with REMS-associated changes in different species through evolution and possibly to predict the functional disorders in association with diseases.

6.7 Confirmation of the Neuro-Physio-Pharmacological Network with Support from REMS-Functional Correlates

Based on the findings from isolated independent studies, it was interpreted that GABA-induced inhibition of the LC-NA-ergic REM-OFF neurons is a necessity for the generation of REMS and non-cessation (continued activity) of the REM-OFF neurons prevents appearance of REMS. For confirmation of such neural network being responsible for maintenance of REMS, we hypothesized that if the LC-NA-ergic REM-OFF neurons were not allowed to cease activity, not only REMS should be reduced but there should be elevated level of NA in the brain and that should induce expression of otherwise REMS-loss associated changes (symptoms). Indeed, infusion of picrotoxin (Pic), a GABA-antagonist, into the LC prevented REMS and induced NA-induced increased Na-K ATPase activity (Kaur et al. 2004), and the latter was comparable to that observed otherwise due to REMSD (Gulyani and Mallick 1993, 1995; Mallick et al. 2000; Mallick and Singh 2011). Finally, to confirm if we downregulated the NA synthesis in the LC neurons using si-/sh-RNA, REMS was increased (Khanday et al. 2016). Additionally, if such rats with downregulated NA were deprived of REMS, the REMSD-associated NA-induced increased Na-K ATPase activity and neuronal apoptosis was prevented (Somarajan et al. 2016). These findings are proof-of-principle and perhaps fit case to extend the knowledge acquired in the laboratory (bench) from basic animal studies and take it to the bedside for human benefit. To prove, the knowledge may be translated (with or without modification) either by downregulating synthesis or release of NA or by preventing the action of NA in the brain to ameliorate REMS-loss-associated symptoms preferably in the humans.

6.8 Evolutionary Significance of REMS

As described above, the loss of REMS induces elevated level of NA in the brain and that causes many (if not all) of the associated acute and chronic symptoms (Mallick and Singh 2011). Interestingly, REMS is present in animals through evolution in different species and throughout life (ontogenically) in the same individual. The question is, if loss of REMS induces elevated NA and that adversely affects the brain, why in evolution has REMS come into existence and is being maintained (i.e., did not get lost) through generations and running millions of years through evolution? We have mentioned above that the NA-ergic REM-OFF neurons are continuously active through waking, slow down during NREMS, and cease activity during REMS. Therefore, it was likely that the level of NA must be highest during waking, reduced during NREMS, and very low during REMS. As a corollary, it was hypothesized that the NA level should be significantly reduced (i.e., the brain should be washed-off of excess NA) during REMS and it should be elevated during REMS-loss (REMSD). Indeed, we confirmed that NA level is least during REMS and it increases during REMSD (Mehta et al. 2017).

Further, we hypothesized that possibly low dose of NA is beneficial, while higher dose is damaging to the brain. We showed that lower dose of NA acts as an antioxidant that protects the brain (an antioxidant compromised organ) from oxidative insults and promotes neuronal growth as well as branching necessary for synaptogenesis (i.e., the plasticity), while higher dose increases their apoptosis, inhibits neuronal growth, and breaks down neuronal branches (Singh et al. 2019; Giri et al., *communicated after revision*). These findings offer cellular and molecular level explanation of REMS and its loss-associated functional gain or loss in terms of neuronal excitability and memory in health and diseases, respectively. Consolidation of these findings led to my hypothesis that ***through evolution, REMS has evolved to maintain the brain level of NA, which then maintains fundamental housekeeping process(es) of the brain*** (Mallick and Singh 2011; Singh et al. 2019).

6.9 Cognitive States, Dream, and REMS: Possible Neurophysiological Mechanism and Their Relationships

The REMS has been objectively identified by the presence of characteristic electrophysiological signals. The dreams appear intermittently during REMS, which has been sometimes termed as active REMS. However, objective criteria for unbiased identification of dream are missing, i.e., dreams can only be known if the dreamer chooses to convey the same and convey correctly to the observer (another person). Additionally, it depends if the dreamer remembers or how much one remembers correctly the dream content, its sequence of events, and so on. Thus, in the absence of defining objective characteristic, the dreams cannot be identified in animals, and humans may face language barrier to explain the dream content. Often the appearance of dream has been synonymously expressed with the expression of REMS; however, still we cannot comment on the content of the dreams. Notwithstanding,

based on personal experiences, it can be said that unlike NREMS and non-dream period of REMS, the dreams are characterized by cognitive expression, which may be an experience in the past (stored memory) or anew one (may be a creative one). Therefore, my hypothesis is that REMS and dreams are related but independent phenomena where the latter gets intermittently triggered and superimposed during REMS. As discussed above, consistent research has made significant progress in our understanding of the neuronal mechanism of regulation and functions of REMS; however, our knowledge about dream is at best, rudimentary. I proposed that during REMS, some REMS-related neurons intermittently become active and trigger some neurons in the brain areas involved with memory and thought processes so that dream is expressed superimposed on REMS. This proposition could be supported by the fact that there are phasic REM-ON neurons, which become intermittently active during REMS. Also, neurons from the brain area where phasic REM-ON neurons are located have been shown to project to several areas in the brain including the ventral tegmentum, amygdala, and SN, which are part of the limbic system and are (directly or indirectly) associated with memory (Genzel et al. 2015), cognitive processes (van der Helm and Walker 2011), and REMS (van der Helm et al. 2011; Yadav et al. 2019). Findings from our recent and ongoing studies show that presumably REM-ON ACh-ergic neurons modulate REMS by activating the SN (Yadav et al. 2019), amygdala (Yadav and Mallick 2022), and ventral tegmental area (under preparation), supporting our contention. These results would form the basis for future confirmatory studies ideally in humans.

6.10 Possible Existence of All-Pervasive, Overwhelming, Fundamental, Background State: The Consciousness

The modern experimental science (and scientists) recognizes three states of consciousness: wakefulness, NREMS, and REMS, and they may be objectively identified by the expression of characteristic electrophysiological signals. It is generally accepted that under normal, healthy conditions, though REMS follows NREMS and the latter follows wakefulness, these states may transit reversibly from one to the other state except wakefulness to REMS. There is no fixed or no proportionate length of time to be spent in a state before one may transit into the other state and the states may partially exist at the same time, i.e., there may be overlap among states. Also, in diseased condition, REMS may follow wakefulness, and proportion of simultaneous expression of more than one state may increase. I argued that perhaps the missing links in the modern classification of states are that although the consciousness has been classified into three distinct and discrete states, it has not conceived several factors including the following: (1) what the fundamental state is, which is partly or completely transformed into other states; (2) whether there is any background state on which these three states exist (get expressed); (3) where the two states would move when the third gets expressed, if these three states are discrete; (4) how these states are connected and communicate with each other to get expressed coherently and dynamically; and (5) if a state is transformed

from one to the other, whether each of the states would carry the same energy level or, if the energy levels are different, how and where the difference of energy between states get accommodated or accounted for (gained or lost). Further, because of the absence of a concept of such basal state, one finds it untenable to explain the overlapping and partial combination of existence of various states be it in health or in diseases. In principle, I argued that there is likely to exist a basal, all-pervasive, overwhelming, fundamental, background state (medium) on which the three classical states, viz., wakefulness, NREMS, and REMS, play around and get expressed. This concept at least helps explaining expression of common intermediary as well as various levels of transitory phases connecting the classical states. It also allows us to conceive how states can be of various combination, how they can overlap to different degrees, and how they can transit easily, completely or partially, in a reversible manner. Although apparently this concept may appear not to satisfy why normally REMS does not appear following waking, my argument against it is as follows: because grossly the EEG, EOG, and apparent cognitive experience (dreams and thoughts) in waking and REMS are comparable (except the voluntary physical acts), one may not be able to differentiate them, which needed subtler characteristic identifiable signals.

My concept of existence of such overwhelming, all-pervasive, basal, fundamental state satisfies the fundamental lacuna and discrepancy raised above. However, it needs to be explained, preferably in a demonstrable manner what the state could be and what should be its characteristic. I would explain with the following analogous conditions as examples and then offer neurophysiological explanation. In a fluid (liquid or gaseous) environment, e.g., pond, river, sea or, in the atmosphere for that matter, there are finer surface ripples of waves of various frequencies and intensities. Due to various quality and quantity (strengths), the inputs get either distributed or absorbed, i.e., indiscernible or aggregate at various location(s), and get expressed over the ripple on the relatively quieter fluidic surface. Such changes may get expressed as local wind, storm, gale, hurricane, etc. Those inputs could arise at various locations within the system or may be due to external influence modulating at different regions (even away from the expression) of the system. Another example could be if we mix up many waves of varying low intensity and frequencies (noise) generated from various generators, a network of frequencies can be created (e.g., a frequency shield). This mixture of networks may be influenced by various inputs from the said generators where some focal island of wave patterns may be created depending on the quality and quantity of inputs. These low, medium, or high energy containing waves (or wavelets) may be compared with the locality and non-locality concept of the quantum physics (details are not being discussed in this chapter). In the examples above, regarding recordable parameter of consciousness, the ripples are the EEG waves (may be the ultralow wave and frequency) due to local cortical neural networks at the cortical surfaces which are least influenced by far and near input(s), i.e., beyond the local basic network. These ripples are influenced by various levels of central and peripheral inputs, often involving the brainstem and thalamic reticular connections, which express synchronization and desynchronization of the

EEG. The local, sub-local, relatively wider, and global changes so generated give rise to wakefulness, NREMS, REMS, and their overlaps as well as variations.

6.11 Support from Ancient *Upanishadic* Literature to Explain Conscious State: The *T-State*

My views mentioned in the preceding paragraphs may be supported, at least conceptually and philosophically. I looked for if the ancient Indian philosophers and sages might have mentioned anything comparable out of their wisdom. The sixth chapter of *Mandukya Upanishad* refers to sleep-waking-dreaming, and in that segment, one finds a mention of a fourth state, called ***Thuriya-state***, which conceptually supports my explanation, although there is no experimental verification. In an attempt to assign an identity to the ripple explained above in my examples, I borrowed the concept of ***Thuriya state*** and termed it as ***T-state***, as the most fundamental, all-pervasive, background state primarily due to the waves created by the local neural networks, due to the local field potentials and for the sustainability of those networks. Various sensory-motor inputs from within and outside the networks give rise to potentials of various dimensions and phases, which are recorded as EEG associated with the three classical conscious states: the REMS and intermittent dreams (the interactions among the neurons in the core and primitive brain areas), the NREMS (the interaction of other neurons in the brain over and above those responsible for inducing REMS), and wakefulness (the strongest of the three apparently discrete states caused due to strong inputs from various parts of the body). Considering these, I have explained that quantitative and duration of masking and unmasking of part of the ***T-state*** describe various discrete or overlapping states during health and diseases (Mallick and Mukhopadhyay 2011); this is a testable hypothesis.

Acknowledgments To all my present students in the lab to go through and comment on an earlier version of this manuscript and helping me by arranging the references. Fundings from JC Bose fellowship and UGC-DRSII are gratefully acknowledged.

References

Alam MN, Kumari S, Mallick BN (1993) Role of GABA in acetylcholine induced locus coeruleus mediated increase in REM sleep. Sleep Res 22:541

Arnulf I (2013) Dream imagery, rapid eye movement sleep behavior disorder, and hallucinations. Sleep Biol Rhythms 11:15–20

Aserinsky E (1999) Eyelid condition at birth: relationship to adult mammalian sleep-waking patterns. In: Mallick BN, Inoue S (eds) Rapid eye movement sleep. Marcel Dekker, New York, pp 1–16

Aserinsky E, Kleitman N (1953) Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science 118:273–274

Aston-Jones G, Bloom FE (1981) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 1:876–886

Berger H (1929) Über das Elektrenkephalogramm des Menschen. Arch Psychiatr Nervenkr 87: 527–570
Dement W (1960) The effect of dream deprivation. Science 131:1705–1707
Egan TM, North RA (1985) Acetylcholine acts on m2-muscarinic receptors to excite rat locus coeruleus neurons. Br J Pharmacol 85:733–735
Genzel L, Spoormaker VI, Konrad BN, Dresler M (2015) The role of rapid eye movement sleep for amygdala-related memory processing. Neurobiol Learn Mem 122:110–121
Gulyani S, Mallick BN (1993) Effect of rapid eye movement sleep deprivation on rat brain Na-K ATPase activity. J Sleep Res 2:45–50
Gulyani S, Mallick BN (1995) Possible mechanism of rapid eye movement sleep deprivation induced increase in Na-K ATPase activity. Neuroscience 64:255–260
Hobson JA, McCarley RW, Wyzinski PW (1975) Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. Science 189:55–58
Hobson JA, McCarley RW, Nelson JP (1983) Location and spike-train characteristics of cells in anterodorsal pons having selective decreases in firing rate during desynchronized sleep. J Neurophysiol 50:770–783
Inglis WL, Winn P (1995) The pedunculopontine tegmental nucleus: where the striatum meets the reticular formation. Prog Neurobiol 47:1–29
Jouvet M (1999) Around the discovery of REM sleep in cats. In: Mallick BN, Inoue S (eds) Rapid eye movement sleep. Marcel Dekker, New York, pp v–ix
Jouvet M, Michel F (1958) [Study of the cerebral electrical activity during sleep]. C R Seances Soc Biol Fil 152:1167–1170
Kaur S, Saxena RN, Mallick BN (1997) GABA in locus coeruleus regulates spontaneous rapid eye movement sleep by acting on GABA-A receptors in freely moving rats. Neurosci Lett 223:105–108
Kaur S, Panchal M, Faisal M, Madan V, Nangia P, Mallick BN (2004) Long term blocking of GABA-A receptor in locus coeruleus by bilateral microinfusion of picrotoxin reduced rapid eye movement sleep and increased brain Na-K ATPase activity in freely moving normally behaving rats. Behav Brain Res 151:185–190
Khanday MA, Somarajan BI, Mehta R, Mallick BN (2016) Noradrenaline from locus coeruleus neurons acts on pedunculo-pontine neurons to prevent REM sleep and induces its loss-associated effects in rats. eNeuro 3(6):ENEURO.0108-16.2016
Kumar R, Bose A, Mallick BN (2012) A mathematical model towards understanding the mechanism of neuronal regulation of wake-NREMS-REMS states. PLoS One 7:e42059
Mahowald MW, Woods SR, Schenck CH (1998) Sleeping dreams, waking hallucinations, and the central nervous system. Dreaming 8:89–102
Mallick BN, Inoue S (eds) (1999) Rapid eye movement sleep. Marcel Dekker, New York, p 419
Mallick BN, Mukhopadhyay AK (2011) REM sleep and dream sleep: are they identical? Exploring the conceptual developments in the Upanishads and the present knowledge based on the neurobiology of sleep. In: Mallick BN et al (eds) Rapid eye movement sleep: regulation and function. Cambridge University Press, England, pp 21–30
Mallick BN, Singh A (2011) REM sleep loss increases brain excitability: role of noradrenaline and its mechanism of action. Sleep Med Rev 15:165–178
Mallick BN, Siegel JM, Fahringer H (1990) Changes in pontine unit activity with REM sleep deprivation. Brain Res 515:94–98
Mallick BN, Thankachan S, Islam F (1998) Differential responses of brain stem neurons during spontaneous and stimulation-induced desynchronization of the cortical EEG in freely moving cats. Sleep Res Online 1:132–146
Mallick BN, Adya HV, Faisal M (2000) Norepinephrine-stimulated increase in Na+, K+-ATPase activity in the rat brain is mediated through alpha1A-adrenoceptor possibly by dephosphorylation of the enzyme. J Neurochem 74:1574–1578

Mallick BN, Kaur S, Saxena RN (2001) Interactions between cholinergic and GABAergic neurotransmitters in and around the locus coeruleus for the induction and maintenance of rapid eye movement sleep in rats. Neuroscience 104:467–485

Mallick BN, Thankachan S, Islam F (2004) Influence of hypnogenic brain areas on wakefulness- and rapid-eye-movement sleep-related neurons in the brainstem of freely moving cats. J Neurosci Res 75:133–142

Mallick BN, Pandi-Perumal SR, McCarley RW, Morrison AR (eds) (2011) Rapid eye movement sleep: regulation and function. Cambridge University Press, London, p 478

Mallick BN, Singh A, Khanday MA (2012) Activation of inactivation process initiates rapid eye movement sleep. Prog Neurobiol 97:259–276

Manni R, Pacchetti C, Terzaghi M, Sartori I, Mancini F, Nappi G (2002) Hallucinations and sleep-wake cycle in PD. Neurology 59:1979

McCarley RW, Hobson JA (1971) Single neuron activity in cat gigantocellular tegmental field: selectivity of discharge in desynchronized sleep. Science 174:1250–1252

McCarley RW, Hobson JA (1975) Neuronal excitability modulation over the sleep cycle: a structural and mathematical model. Science 189:58–60

Mehta R, Singh S, Khanday MA, Mallick BN (2017) Reciprocal changes in noradrenaline and GABA levels in discrete brain regions upon rapid eye movement sleep deprivation in rats. Neurochem Int 108:190–198

Moruzzi G (1972) The sleep-waking cycle. Ergeb Physiol 64:1–165

Pal D, Mallick BN (2004) GABA in pedunculo pontine tegmentum regulates spontaneous rapid eye movement sleep by acting on GABAA receptors in freely moving rats. Neurosci Lett 365:200–204

Pal D, Mallick BN (2006) Role of noradrenergic and GABA-ergic inputs in pedunculopontine tegmentum for regulation of rapid eye movement sleep in rats. Neuropharmacology 51:1–11

Pal D, Mallick BN (2009) GABA in pedunculopontine tegmentum increases rapid eye movement sleep in freely moving rats: possible role of GABA-ergic inputs from substantia nigra pars reticulata. Neuroscience 164:404–414

Rechtschaffen A, Kales A (eds) (1968) A manual for standardized terminology, techniques and scoring system for sleep stages of human subjects. Issue 204 of NIH Publication; U. S. National Institute of Neurological Diseases and Blindness, Neurological Information Network; UCLA, Brain Information Service, NINDB Neurological Information Network (U.S.)

Sakai K (1988) Executive mechanism of paradoxical sleep. Arch Ital Biol 126:239–257

Siegel J (1989) Brainstem mechanisms generating REM sleep. In: Kryger M et al (eds) Principles and practice of sleep medicine. Saunders, Philadelphia, pp 104–120

Singh S, Mallick BN (1996) Mild electrical stimulation of pontine tegmentum around locus coeruleus reduces rapid eye movement sleep in rats. Neurosci Res 24:227–235

Singh A, Das G, Kaur M, Mallick BN (2019) Noradrenaline acting on alpha1 adrenoceptor as well as by chelating iron reduces oxidative burden on the brain: implications with rapid eye movement sleep. Front Mol Neurosci 12:7. https://doi.org/10.3389/fnmol.2019.00007

Somarajan BI, Khanday MA, Mallick BN (2016) Rapid eye movement sleep deprivation induces neuronal apoptosis by noradrenaline acting on alpha1 adrenoceptor and by triggering mitochondrial intrinsic pathway. Front Neurol 7:25. https://doi.org/10.3389/fneur.2016.00025

Steriade M, McCarley RW (eds) (2005) Brain control of wakefulness and sleep. Kluwer Academic/Plenum, New York

Thankachan S, Islam F, Mallick BN (2001) Role of wake inducing brain stem area on rapid eye movement sleep regulation in freely moving cats. Brain Res Bull 55:43–49

van der Helm E, Walker MP (2011) Sleep and emotional memory processing. Sleep Med Clin 6:31–43

van der Helm E, Yao J, Dutt S, Rao V, Saletin JM, Walker MP (2011) REM sleep depotentiates amygdala activity to previous emotional experiences. Curr Biol 21:2029–2032

Williams JA, Reiner PB (1993) Noradrenaline hyperpolarizes identified rat mesopontine cholinergic neurons in vitro. J Neurosci 13:3878–3883

Yadav, R. K. and Mallick, B. N., Dopaminergic- and cholinergic-inputs from substantia nigra and pedunculo-pontine tegmentum, respectively, converge in amygdala to modulate rapid eye movement sleep in rats, Neuropharmacology, 2022 (in press). https://doi.org/10.1016/j.neuropharm.2021.108607

Yadav RK, Khanday MA, Mallick BN (2019) Interplay of dopamine and GABA in substantia nigra for the regulation of rapid eye movement sleep in rats. Behav Brain Res 376:112169. https://doi.org/10.1016/j.bbr.2019

Novel Cellular Stress Models with Implications in Understanding and Treating ENT Pathologies

7

Naresh Kumar Panda, Maryada Sharma, Anurag Snehi Ramavat, Sourabha Kumar Patro, Gyan Ranjan Nayak, and Kavita Kaushal

Abstract

Interaction with the world around us requires extracting meaningful signals to guide behaviour. The mammalian senses of olfaction, vision, somatosensation, hearing, balance and taste facilitate extraction of sense-specific information. Most sensory organs in the vertebrate head originate from cranial placodes (CPs). CPs are formed embryonically through a series of differentiation steps arising at the boundary between neural and non-neural ectoderm, and they can be divided into anterior, posterior and intermediate groups depending on their place of origin in the developing embryonic head. Anterior CPs include adenohypophyseal, olfactory and lens placodes; intermediate CPs include the trigeminal placode, which gives rise to the sensory neurons of the ophthalmic and maxilla-mandibular divisions of the trigeminal ganglion; posterior CPs are comprised of the otic, lateral line placode and the epibranchial placodes that give rise to the inner ear, lateral line organs (in fish and amphibian) and sensory neurons of the geniculate, petrosal and nodose ganglia, respectively. The complexity of neural plate border specification in vitro poses a major limitation to gain deeper mechanistic insights into the developmental cues driving efficient placodal differentiation; hence generation and establishment of in vitro cellular models with improved cranial placode differentiation are challenging. Our group is interested in the establishment of cranial/sensory placodes in vitro using novel cellular stress stem cell reprogramming models with translational implications in sensorineural hearing loss regeneration and modelling COVID-19-associated anosmia. We are primarily interested in building the otic placodes that can form viable otic vesicles

N. K. Panda · M. Sharma (✉) · A. S. Ramavat · S. K. Patro · G. R. Nayak · K. Kaushal
Department of Otolaryngology and Head and Neck Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India
e-mail: sharma.maryada@pgimer.edu.in

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_7

in vitro, which can be further directed to generate cochlear/vestibular systems of inner ear and the sensory neurons of its associated vestibulocochlear ganglion. Given the copious involvement of serine proteases in COVID-19 pathogenesis, we are also encouraged to leverage our proteolytic stress cellular models towards establishment and characterization of novel olfactory epithelial neurospheres housing supporting cells, progenitor cells and sensory neurons for investigating cellular and molecular targets of COVID-19-associated anosmia.

Keywords

Cochlear system · Vestibulocochlear ganglion · Reprogramming models · Sensory neurons · Hearing loss regeneration

7.1 Introduction

Sensorineural hearing loss (SNHL) is a neurological disability that impacts the physical, developmental, cognitive and socio-economic well-being of patients (Contrera et al. 2016; Roland Jr et al. 2016; Li et al. 2015; Tseng et al. 2016; Boulet et al. 2009; Stevenson et al. 2010; Niclasen et al. 2016; Olusanya et al. 2014). A considerable fraction (over two-thirds) of adults 70 years or older in the United States suffer clinically meaningful hearing loss (Goman and Lin 2016; Lin et al. 2011). Alarmingly, hearing loss is posed as a potential risk factor for accelerated cognitive impairment in the elderly associated with increased social isolation, anxiety and depression (Curhan et al. 2020; Jayakody et al. 2018; Lawrence et al. 2018, 2020; Cosh et al. 2019; Huber et al. 2020). Hearing loss can result in significant communication disorders and lack of speech acquisition and cognitive, social and emotional development in children as it affects nearly 1 in 1000 newborns; clinically relevant hearing loss evaluation may vary depending on diagnostic criterion, screening techniques and follow-up studies (Morton and Nance 2006). Importantly, though the heterogeneity confounds diagnosis in hearing loss, however, it also positions hearing specialists with a potential to offer effective individualized/customized treatments for genetic-deaf patients (Rudman et al. 2018), given the genetics has been well established.

Human cochleae acquire functional maturation neonatally; therefore, mature mammalian cochleae are nonregenerative by design—presenting one of the most difficult challenges to address. A long-standing challenge in the hearing loss regeneration field is the development of faithful models for deriving functional cochlear hair cells from human pluripotent stem cells (hPSCs) (Chen et al. 2012, 2016; Tang et al. 2016; Ohnishi et al. 2015; Ealy et al. 2016; Ronaghi et al. 2014). The protocols described thus far employ complex methods to steer stem cell differentiation towards HC (hair cell) fate by mimicking in vivo HC developmental/signalling pathways (Oshima et al. 2010; Koehler et al. 2013). Despite recent possible efforts in hearing loss regenerative research (McLean et al. 2017; Walters et al. 2017; Warnecke et al. 2017; Schaefer et al. 2018; Perny et al. 2017; Duran Alonso et al. 2018; Meas et al.

2018), the inner ear hair cell-like phenotypes displaying maturation markers and functional electrophysiology have not been achieved till date (Koehler et al. 2017; Munnamalai and Fekete 2017). However, further refinements (molecular/cellular manoeuvring) to the protocol given by Koehler et al. (2017) are suggested to diversify the generation of inner ear cell types and may result in recreation of cochlear hair cells (in addition to vestibular hair cells) within the organoid. Accordingly, there is an unmet need for replicable, scalable, efficient and xenogeneic-free methods for coaxing human pluripotent stem cells into inner ear organoids that are suitable for clinical cell therapies. The success in generation of mature inner cochlear hair cells will provide for an opportunity for quantitative in vitro disease modelling, drug screening applications and cell-based transplantation therapy in sensorineural hearing loss patients to restore appreciable hearing.

Reduction of smell is now recognized as one of the cardinal symptoms of COVID-19. The deficit appears to be most often transient and recovers after several days to weeks. Anosmia in COVID-19 differs from other virus-associated deficits. It is sudden in onset with rapid recovery. We have come to realize that to understand COVID-19, it is necessary to consider multiple dimensions, from the cellular-molecular level to clinical features relating different aspects of the disease in a holistic approach that has been lacking. Integrating multiple disciplines provides a more complete insight into the pathogenesis of the disease process. Anosmia and hypogeusia were not initially recognized to be linked to COVID-19; they were mentioned to affect only about 5% of COVID-19 patients in one of the first studies from China (Mao et al. 2020), but a much higher prevalence was reported in subsequent studies from Europe, the Middle East and North America (Agyeman et al. 2020; Hannum et al. 2020; Passarelli et al. 2020; Printza and Constantinidis 2020; Sedaghat et al. 2020; Tong et al. 2020; von Bartheld et al. 2020). Key to understanding such differences in infectivity of SARS-CoV-2 may lie in the frequency of variants in the virus entry proteins, ACE2 and TMPRSS2, which may depend on cell type and population, with implications for infectivity, virus spread and therefore management of the COVID-19 pandemic. It has been a major mystery how the virus affects the senses of smell. Significant progress has now been made to begin to elucidate the cellular and molecular mechanisms of coronavirus-induced anosmia. Recent work has provided new insights into the cell types in the olfactory epithelium that express the relevant virus entry proteins (Bilinska and Butowt 2020) and that accumulate the virus after infection (Bryche et al. 2020). In the olfactory epithelium, the evidence suggests a distinct cascade of cellular events that can explain the transient anosmia in COVID-19. In this context, we explain the importance of developing novel in vitro models for future research in this field. We support the growing literature that proposes anosmia seen in COVID-19 as an early, rapid and surprisingly effective diagnostic screening tool.

7.2 Implications of Novel Patient-Specific Culture Models for Elucidating Genotype-Phenotype Crosstalk for Sensorineural Hearing Loss Regeneration

7.2.1 Current Challenges in Hearing Loss Diagnosis and Clinical Decision-Making

Aetiology of Hearing Loss: Hearing loss is the most common sensory deficit with over 432 million adults and 34 million children affected by this disability, thereby posing it as a major health and economic burden on society. The World Health Organization estimates are alarming, and the number of hearing loss sufferers are predicted to go over 900 million by 2050 (World Health Organization n.d.). Approximately half of these cases have an underlying genetic basis for their HL (hearing loss). Genetic hearing loss displays extraordinary allelic, genetic, phenotypic and clinical heterogeneity, and almost 80% of familial HL is nonsyndromic hearing loss (NSHL). Currently, more than 150 NSHL genes have been identified (https://hereditaryhearingloss.org), which encode for a variety of proteins that have diverse underlying pathogenic mechanisms leading to hearing loss (Müller and Barr-Gillespie 2015; Carpena and Lee 2018). There are 876,135 genetic variants found within the 152 known nonsyndromic and syndromic deafness genes, of which 7474 are classified as pathogenic, implying a causal association with deafness (http://deafnessvariationdatabase.org (Azaiez et al. 2018)).

Genetic Basis and Aetiology of Hearing Loss in Indian Population: The Indian Council of Medical Research (ICMR) estimates for HL prevalence in urban areas in India are 6.8%, with nearly 30,000 infants affected with congenital hearing loss in India each year (Yan et al. 2015). The estimated prevalence of adult-onset deafness is 7.6% and childhood-onset is 2% (Garg et al. 2009). The genetic data for hearing loss is limited for North Indian patients visiting our centre (ENT department, PGIMER, Chandigarh), and low incidence of GJB2, GJB6 and mitochondrial DNA mutations in NSHL had been reported (Bhalla et al. 2009, 2011). Majority of the studies from India are based on consanguineous South Indian population and Muslims families (from J&K), which exhibit higher incidence of hereditary hearing loss involving pathogenic GJB2 mutation in South India (Arunachalam et al. 2020) OTOF, CLDN14 and SLC26A4 mutations (Pandey et al. 2017) and novel OTOF pathogenic variant in whole exome sequencing screen (Kuchay et al. 2020) in Jammu & Kashmir, India. High allelic heterogeneity has been reported for mutations in TMPRSS3, TMC1, USH1C, CDH23 and TMIE (Ganapathy et al. 2014).

Etiological Heterogeneity and Limitations of Current Diagnosis: The etiological diversity underlying hearing loss poses a massive challenge to efficient diagnosis and treatment. The routine hearing loss detection including ABR, OAE, etc. is acceptable; however, advance hearing loss diagnostic approaches consider audioprofiles supportive in counselling when a genetic diagnosis has already been established. Audioprofiles do not faithfully represent pathogenic variants for certain mutations associated with spectra of phenotypes (Kremer 2019), and remarkable intrafamilial variability is reported for POU4F3 (DFNA15) (Kitano et al. 2017;

Pauw et al. 2008; Vahava et al. 1998) and MYO6 (Oonk et al. 2013; Sanggaard et al. 2008; Topsakal et al. 2010). Moreover, the implications of missed and delayed diagnoses indicate a room for improvement. Efforts to identify genes responsible for HL have been challenged by high genetic heterogeneity and different ethnic-specific prevalence of inherited deafness.

7.2.2 Genetic Implications for Cochlear Implantation

National Scenario: Cochlear implants (CIs) are used in congenital and later onset deafness and currently serve as standard tools to treat SNHL in children and have shown benefits for spoken language, reading skills and cognitive improvement (Niparko et al. 2010). However, post-CI hearing outcomes may vary depending on the genetic aetiology and various other factors. Surgical intervention with CI at a younger age has been correlated with better prognosis in Indian children with severe-to-profound hearing loss (Hota 2019); however, genetics underlying CI outcomes is completely unexplored in Indian population. The National Program for Prevention and Control of Deafness (NPPCD) and the Cochlear Implant Group of India (CIGI) have successfully streamlined CI funding with over 20,000 CI surgeries carried out in India since 1996 and sponsorship of free CI to all children below 6 years in several states. The State of Tamil Nadu (with highest prevalence of HL) has opened up innovative satellite centres for improving habilitation outcomes (Kumar and Kameswaran 2019). CI workups are extremely expensive to afford, and a (otherwise preventive) failed outcome tends to have huge monetary setbacks to the government, sponsoring agency or the individual cost-bearing families.

International Scenario: Importantly, gene-specific post-CI outcomes have been reported for very few mutations including GJB2 and SLC26A4 and with varied results (Green et al. 2002; Matsushiro et al. 2002; Bauer et al. 2003; Sinnathuray et al. 2004; Cullen et al. 2004; Wu et al. 2008, 2011a; Karamert et al. 2011; Yan et al. 2013; Yoshida et al. 2013; Davcheva-Chakar et al. 2014). Early intervention with CI in patients with GJB2 and SLC26A4 mutations is associated with good post-implant outcomes (Wu et al. 2015). Other mutations that have been associated with good CI outcomes include those in OTOF (Rouillon et al. 2006; Wu et al. 2011b; Zhang et al. 2013), COCH (Vermeire et al. 2006; Tsukada et al. 2015), MYH9 (Nishiyama et al. 2013; Pecci et al. 2014; Canzi et al. 2016), MYO6 (Volk et al. 2013; Miyagawa et al. 2015), MYO15A (Miyagawa et al. 2013, 2015), TECTA (Miyagawa et al. 2013), CDH23 (Usami et al. 2012), TMPRSS3 (Miyagawa et al. 2015; Elbracht et al. 2007; Weegerink et al. 2011)) and mitochondrial mutations. However, POU3F4 mutations and genetic variants in SGNs are correlated with worse CI outcomes (Lee et al. 2009; Stankovic et al. 2010; Miyagawa et al. 2016; Pollak et al. 2016; Shearer et al. 2017).

Scope and Importance of Investigating Gene-Specific CI Outcomes: High-throughput NGS technology has revolutionized clinical data acquisition and analyses of correlations between genotypes and outcomes of CI rehabilitation (Wu et al. 2013, 2015; Miyagawa et al. 2013, 2015; Nishio and Usami 2015). Importantly, similar studies that could provide genetic information about CI

outcomes and facilitate clinical decision-making between cochlear implantation and rehabilitation are lacking for Indian population. Therefore, studies that define gene-specific CI outcomes can certainly lead to more efficient and individualized treatment strategies. NGS HL gene panels will increasingly allow for etiological diagnosis in hereditary hearing loss patients, and with integration of such genetic tests for HL into routine clinical practice, patients can more rapidly obtain an accurate diagnosis and prediction outcomes for CI, thereby avoiding expensive CI workups.

Establishing Genetic Testing for Hearing Loss Diagnosis and Developing Potential Therapeutic Targets: Establishing a genetic diagnosis of HL is of great significance for clinical evaluation of patients, estimating recurrence risks for their families, clinical decision-making and developing patient-specific potential therapeutic targets for precision medicine (Rudman et al. 2018; Carpena and Lee 2018; Azaiez et al. 2018; Kremer 2019; Vona et al. 2016; Hao et al. 2018). The emerging success of next-generation sequencing (NGS) and identification of novel mutations has put it on forefront of faithful hearing loss diagnosis approach and has furthered the field by defining the mutational landscape of hearing loss, thereby driving the discovery of novel gene-phenotype relationships (Azaiez et al. 2014; Booth et al. 2018). Rigorous NGS-based gene panels for hearing loss are currently employed for diagnosis and research. The Otological Sequence Capture of Pathogenic Exons (OtoSCOPE) panel, developed by the University of Iowa in 2010, has been extensively used since then to identify causative mutations underlying hearing loss (Azaiez et al. 2014, 2015; Shearer et al. 2010, 2014; Mori et al. 2015; Sakuma et al. 2015; Sloan-Heggen et al. 2016). Other NGS panels for hearing loss include OtoGenomeTM (Fedick et al. 2016), OtoChip, OtoSeq, Hereditary Hearing Loss APEX test, custom-design NGS gene panels (Booth et al. 2015) and MiamiOtoGenes (Tekin et al. 2016). NGS has exposed the extraordinary allelic heterogeneity underpinning hearing loss and reported more than 876,000 variants, of which ~7000 are disease causing across deafness-associated genes (Azaiez et al. 2018). The massive data for rare variants necessitates the need to further characterize the identified variants for meaningful interpretations and development of viable therapies.

7.3 Unravelling Genotype-Phenotype Complexities Underlying Hearing Loss and Coupling Regenerative Therapies for Patient-Specific Individualized Treatment Options

Potential of In Vitro Models in Hearing Loss Regeneration Research: In the emerging era of precision medicine, organoids have offered intensive investigations for disease modelling and high-throughput drug screening. Success in establishment of faithful inner ear organoids aimed at tailoring interventions for inner ear pathologies would be immensely useful in investigating treatments for sensorineural hearing loss, vestibular dysfunction and cell-based transplantation therapy for the

same. Employing patient-derived induced pluripotent stem cells for generating inner ear organoids could be useful to study the underlying molecular mechanisms that lead to the clinical phenotypes. These in vitro model systems offer a remarkable platform for testing ototoxicity that is traditionally conducted using animal models; such animal studies suffer the limitation of modest to no correlation with the susceptibility of human patients to ototoxic agents (Forge and Schacht 2000; Wu et al. 2001; Ding et al. 2016).

Potential Inner Ear Regeneration Approaches: Establishment and detailed characterization of an appropriate in vitro model (which could closely approximate inner ear development and function) would facilitate effective investigation of various genetic and pharmacologic interventions for hearing loss and vestibular dysfunction restoration (Park et al. 2015). Besides that, the other potential near-future applications include cell-based transplantation therapies for hearing loss (Chen et al. 2012). The use of stem cell-based approaches for generation of inner ear components started a while back with the human pluripotent stem cells (hPSCs), and the first structurally identifiable hair cell-like cells (which resembled vestibular hair cells but not the auditory hair cells) were generated from mouse embryonic stem cells (ESCs) (Oshima et al. 2010). Besides employing exogenous pluripotent stem cells for generating inner ear organoids/tissues (Koehler et al. 2017), the recent studies that are actively geared towards addressing hearing loss include the manipulation of signalling and transcription factors that drive neuronal and hair cell fates (Gálvez et al. 2017) and the use of growth factor and drug-based cocktails (co-manipulating Wnt and Notch pathways) to expand and differentiate Lgr5+ inner hair progenitor (subpopulation of cochlear supporting cells) cells into Myo7A/MYO7A $^{+}$ hair cells for cochlear homeostasis and mechanics (McLean et al. 2017). This approach holds a potential for therapeutically converting Lgr5+ cells to inner hair cells in situ. The precise anatomy of the cochlea dictates the articulated cochlear mechanics; therefore, in situ targeting of endogenous stem cells is a promising approach for hearing loss restoration. Human cochleae acquire functional maturation neonatally; therefore, mature mammalian cochleae are nonregenerative by design—presenting one of the most difficult challenges to address. However, a recent study (Walters et al. 2017) has encouraged regenerative approaches for rehabilitation of acquired and/or age-related hearing following SNHL, by overcoming the aging-imposed caveats on reprogramming of adult cochlear cells. They successfully demonstrated phenotypic conversion of non-sensory supporting (Deiters' and Pillar) cells to "new" cochlear hair cells (Myo7A+) in adult mice by co-manipulation of ATOH1 and p27kip1.

The major approaches to treat hearing loss using contemporary stem cell-based therapy include the following methods:

(A) Use of survival/neuroprotective factors to protect non-damaged cochlear progenitor cells.
Regeneration with endogenous stem cells (Lgr5+ supporting cells)

(B) Regeneration with exogenous stem cells (mesenchymal, human embryonic and human-induced pluripotent stem cells)
(C) Regeneration by establishing inner ear organoids/tissues using iPSCs derived from normal and diseased subjects
  (a) Normal iPSCs (with the goal of long-term cell-based transplantation therapy)
  (b) Genetically deaf patient-derived iPSCs (with the goal of correcting/editing mutations implicated in hearing disorder to restore hearing function in the long term)

Massive focus is directed towards (1) generating mature/functional inner hair cells and (2) re-establishing a connection between auditory neurons and inner hair cells to restore hearing loss.

Current Approaches and Challenges in Hearing Loss Regeneration Research: The molecular and genome editing/CRISPR strategies are now being coupled to regenerative/stem cell approaches to address patient-specific hearing loss restoration (Tang et al. 2016; McLean et al. 2017; Fukunaga et al. 2016; Fuster-García et al. 2017). The current therapeutic approaches (genetic, molecular and regenerative) to treat sensorineural hearing loss hold a great promise to be transformative in the long run; however, up until date, they remain far from optimal in restoring complex auditory functions (Landegger et al. 2017). Therefore, there is an unmet need for improved regenerative therapies in the inner ear hearing loss treatment as it affects nearly 40 million adults and is projected to increase with persistent increase in noisier surroundings (https://www.nidcd.nih.gov/), extended longevity of the super-aged societies and unsatisfactory success with current approaches (Müller and Barr-Gillespie 2015; Géléoc and Holt 2014). With the improvement in our understanding and recent progress towards identification of the intrinsic and extrinsic molecular/cellular signalling pathways, there is a hope to re-establish stemness in the mammalian cochleae. Moreover, identification of cochlear supporting cells as inner hair cell progenitors (Shi et al. 2012) has extended molecular (McLean et al. 2017) and genetic (Walters et al. 2017) options that exist for re-establishing regenerative capacity in the adult mammalian cochlea (Lee et al. 2018; Gao et al. 2017; Zhang et al. 2018).

In Vitro Models for Hearing Loss Regeneration (Current Scenario): The current focus of inner ear regeneration research is on the understanding and establishment of successful inner ear developmental models (Perny et al. 2017; Duran Alonso et al. 2018; Gálvez et al. 2017; Chacko et al. 2020; Ogier et al. 2019; Czajkowski et al. 2019; Elliott et al. 2018; Takeda et al. 2019; Thomas and Raible 2019; Fritzsch et al. 2015, 2019; Raft and Groves 2015; Hoijman et al. 2017; Guo et al. 2018; Zhong et al. 2019; Ritter and Martin 2019; Tucker et al. 2018; Fettiplace and Nam 2019; Dyballa et al. 2017; Tang et al. 2019; Hartwell et al. 2019; Bardhan et al. 2019; Walters and Cox 2019; Defourny 2019; Schilder et al. 2019; Lewis et al. 2018; Yamahara et al. 2019) and generation of otic progenitors (Lenz et al. 2019; Lopez-Juarez et al. 2019; Roccio et al. 2018; Lahlou et al. 2018; Chen et al. 2018; Senn

et al. 2020; Xia et al. 2019; McGovern et al. 2019) and inner ear organoids (Roccio and Edge 2019) comprising of sensory epithelia with inner hair cells under in vitro conditions. The protocols for generating the inner ear progenitors had been in practice for a while (Oshima et al. 2010; Li et al. 2003; Rivolta et al. 2006; Hu et al. 2012); however, the area of establishment and characterization of inner ear organoids is relatively recent (Koehler et al. 2013, 2017; Longworth-Mills et al. 2015; Koehler and Hashino 2014).

A bunch of recent studies have re-emphasized the importance of preservation of inner hair cell stereocilia structural arrangement (staircase morphology, actin core stability) and mechanotransductional functions for sensory hearing; importantly, various mutations in stereocilia have been linked to HL (Corns et al. 2018; Vélez-Ortega and Frolenkov 2019; Tadenev et al. 2019; Blanco-Sánchez et al. 2018; Liu et al. 2018; Mehregan et al. 2019; Ebrahim et al. 2016; Ellwanger et al. 2018; Cunningham and Müller 2019; Geng et al. 2018; Matsuoka et al. 2019; Maoiléidigh and Ricci 2019; Dunbar et al. 2019; Roy and Perrin 2018; Nist-Lund et al. 2019). Investigating the crucial players underlying stereocilia building, maintenance, repair and function can largely contribute to the restoration of hearing loss therapies.

Regenerative Therapy Coupled to Gene-Editing Technology for Restoration of Hereditary Hearing Loss: The genetic editing of iPSCs derived from patients with sensorineural hearing loss (caused by hereditary factors) is a promising method for its treatment. The correction of gene mutations in iPSCs for restoration of hearing loss is a promising tool to treat SNHL. ESCs/iPSCs have been differentiated into connexin (CX) CX37/40/43/45-expressing cells and CX26-expressing cells (Fukunaga et al. 2016). These in vitro models should be useful for establishing inner ear cell therapies and drug screening that target connexin-mediated hearing loss. Similarly, genetic deafness due to the mutation in MYO7A was corrected using CRISPR/Cas9 (Tang et al. 2016). Thus, the studies targeted towards genetic editing of mutated genes underlying SNHL provide insights into the pathogenesis of SNHL and also facilitate design of personalized therapeutic interventions against monogenic diseases by employing patient-specific iPSC organoids/models. Despite the extensive information existing on the genetics of hearing loss, many new insights are yet to be unravelled as the list of genetic mutations underlying deafness is increasing with the growing interest in evolution and development of inner ear and regeneration of inner ear hair cells, spiral ganglion and supporting cells, which are integral components of inner ear and hearing biology. Investigating the causes of hearing loss has implications for genetic counselling, prognosis and for developing future therapies. Mutational and phenotypic spectra have been clearly defined for defects of TMC1 (Nakanishi et al. 2014; Gao et al. 2015; Hassan et al. 2015; Wang et al. 2020a; Zhao et al. 2014; Pan et al. 2018), yet clear genotype-phenotype correlations are far from complete for several deafness-associated genes (Eisenberger et al. 2018).

7.4 Implications of Novel Olfactory Epithelial Neurosphere Culture Models for Investigating Molecular and Cellular Targets Underlying COVID-19-Associated Anosmia

Current Challenges in COVID-19 Therapeutics: Continuous efforts to find potential treatments and understand the molecular determinants of COVID-19 pathogenesis are underway (Wang et al. 2020b; Shen et al. 2020; Liu et al. 2020). Primarily efforts are geared towards understanding the host immune responses (Fung and Babik 2020). Transcriptomic, proteomic and bioinformatic approaches are providing insights to identify potential themes underlying the disease pathways (Grifoni et al. 2020; Xiong et al. 2020; Bojkova et al. 2020; Bock and Ortea 2020). COVID-19 is commonly complicated with coagulopathy (Yin et al. 2020; Zhu et al. 2020); anticoagulants showed some promise in a case series (Negri et al. 2020). Moreover, neutrophils and neutrophil extracellular traps (NETs) (Wang et al. 2020c; Mo et al. 2020; Qin et al. 2020; Fox et al. 2020; Yao et al. 2020) are implicated in COVID-19 (Barnes et al. 2020). Striking similarities emerging between the clinical presentation of severe COVID-19 patients to the known NETopathies including ARDS and microthrombosis have recently been reported (Barnes et al. 2020). However, current NET-targeting drugs are either in preclinical studies or have not been very successful in clinical trials. Therefore, repositioning of old drugs is highly encouraged in disease outbreaks like COVID-19 that need an early intervention (Li and De Clercq 2020).

Disease Pathogenesis and Anosmia: Mechanistically, the near-obligatory dual proteases including angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) underlie productive viral infection in the primary mucosal cellular targets including nasal passage and lung alveoli. Thus, elevated expression of ACE2 and TMPRSS2 heightens invading capacity of SARS-CoV-2. Patients with coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), display extrapulmonary symptoms including chemosensory impairment. Co-expression of ACE2 and TMPRSS2 proteins is reported in the aerodigestive tissues including taste buds of the tongue, nasal epithelium, trachea, bronchioles and alveoli with varying degrees of expression, hence explaining the reported clinical symptoms of COVID-19, such as the loss of taste (dysgeusia), loss of olfaction (anosmia) and respiratory dysfunction. Peripheral nervous system involvement results in anosmia and dysgeusia that are the most frequently reported symptoms specific to early stages of COVID-19 and perhaps useful diagnostic markers (Cooper et al. 2020), with reported prevalence varying widely, from nearly 5% among patients hospitalized in Wuhan, China (Mao et al. 2020) to approximately 88% reported by a multicentre study conducted in Europe (Lechien et al. 2020). Interestingly, anosmia underlying COVID-19 presents strikingly without significant rhinorrhoea or nasal congestion unlike the endemic coronaviruses or other common cold-causing agents. Emerging studies indicate changes related to smell and taste as the predominant neurological symptom during COVID-19. Nearly 80% or more of COVID-19 patients experience anosmia, hyposmia, ageusia, dysgeusia or chemesthesis (Giacomelli et al. 2020; Kaye et al.

2020; Parma et al. 2020; Spinato et al. 2020; Yan et al. 2020). Self-perceptions to chemosensory changes can strongly predict if a subject will test positive for SARS-CoV-2 (Bénézit et al. 2020; Fontanet et al. 2020; Moein et al. 2020; Shweta et al. 2020), and anosmia and dysgeusia have come up to be more predictive than all other symptoms, including fatigue, fever or cough (Menni et al. 2020). The subjective normal chemosensory reporting in COVID-19 patients is not accurately estimated as these patients actually display heightened odour detection thresholds as have been documented in recent studies (Moein et al. 2020; Hornuss et al. 2020; Iravani et al. 2020). Therefore, qualitative and quantitative battery of smell testing using scratch-and-sniff cards or common kitchen items are proposed as potential screening tools for COVID-19 (Iravani et al. 2020; Rodriguez-Morales et al. 2020).

COVID-19-associated anosmia is reported as the first symptom by several patients, and lack of associated rhinorrhoea or nasal congestion is highly suggestive of local inflammatory changes underlying pathogenesis (Giacomelli et al. 2020; Kaye et al. 2020; Parma et al. 2020; Spinato et al. 2020; Vaira et al. 2020) which is further validated to some extent by recent imaging studies of the olfactory system in COVID-19 patients that reveal no to focal inflammation (Eliezer et al. 2020; Galougahi et al. 2020). Therefore, the sensitivity of anosmia as a predictor of COVID-19 in patients in the absence of other nasal symptoms (Haehner et al. 2020; Shoer et al. 2020) is becoming appreciably recognized. The rapid resolution of anosmia over weeks compared to prolonged (in months timescales) olfactory recovery seen in relatively benign endemic coronaviruses or other post-viral olfactory loss, after initial symptoms develop, and no indication of parosmia suggest unique mechanisms of infection by SARS-CoV-2 virus (Lechien et al. 2020; Kaye et al. 2020; Parma et al. 2020; Yan et al. 2020; Hopkins et al. 2020).

The nasal respiratory epithelium (RE) has high expression of SARS-CoV-2 entry genes (like ACE2 and TMPRSS2) than RE lining the trachea or lungs (Hou et al. 2020; Sungnak et al. 2020), and recent studies in macaques, ferrets and cats highlight nasal epithelium as the major source of viral RNA following SARS-CoV-2 infection (Munster et al. 2020; Shi et al. 2020). Therefore, nasal epithelium is proposed to be a major reservoir for the virus (Cooper et al. 2020). The sensory detection occurs in the olfactory epithelium (OE forms 5% of the RE) that is located in the superior-most regions of the nasal epithelium. The OE is a complex chemosensory tissue housing multiple cell types, including immature and mature olfactory sensory neurons (OSNs), non-neuronal cell types, such as the sustentacular cells (SCs), Bowman's gland and microvillar cells and stem cells including globose and horizontal basal cells. SCs reside in close proximity to OSNs that enable odour detection (Liang and Acharya 2020). Interestingly, recently published studies in mouse and human investigating the ACE2 and other viral entry gene expression profiles in OE (Brann et al. 2020; Chen et al. 2020; Fodoulian et al. 2020; Ziegler et al. 2020) present a consensus that reports lack of ACE2 expression in OSNs. Further, co-expression of ACE2 and TMPRSS2 was reported in supporting cell including the SCs, Bowman's gland and microvillar cells and the stem cells that repopulate the epithelium following damage. Human OE studies in COVID-19 patients also

suggest sustentacular cells positivity of SARS-CoV-2 as opposed to OSNs (Cantuti-Castelvetri et al. 2020; Meinhardt et al. 2020).

Localized inflammation in the epithelium is proposed as mechanistic route to explain COVID-19-associated anosmia that might block the olfactory clefts (Eliezer et al. 2020). Alternatively, local increases in inflammatory intermediates including cytokines may influence OSN function in a non-cell autonomous manner as demonstrated by elevated levels of inflammatory cytokines in the OE of infected patients. Inflammatory intermediates have been suggested to indirectly lower the expression of odorant receptor (OR) genes by OSNs resulting in decreased odour perception; interestingly the OR expression levels return to normal following cessation of the inflammatory cascades (Rodriguez et al. 2020). Support cells damaged by virus can potentially change ion gradients and fuel availability, thereby altering the OSN firing rates. SCs anatomically support OSN sensory cilia and, therefore, explain the chemosensory deficits even in the absence of direct OSN infection (Bryche et al. 2020). The horizontal basal cells are normally quiescent; however, they play an important role in regenerating damaged OE; however, they also express ACE2 and are highly vulnerable to viral infection (Brann et al. 2020).

7.5 Mechanisms Behind Anosmia and COVID-19 Disease Severity

The investigation of possible mechanisms underlying chemosensory changes in COVID-19 disease has been gaining increasing attention following the significant correlation between anosmia and COVID-19 severity and high viral loads in nasal passage correlating to inflammatory episodes in blood (Bilinska and Butowt 2020; Butowt et al. 2020a,b; Pezzini and Padovani 2020; Saussez et al. 2020; Torabi et al. 2020). However, much remains to be learned about the pathophysiology of SARS-CoV-2 that triggers anosmia. The emerging studies propose that SARS-CoV-2 cell entry genes ACE2 and TMPRSS2 are abundantly expressed in the olfactory, gustatory and chemesthetic systems, including epithelial, support and stem cells responsible for maintaining perception, but they are not expressed in primary or secondary neurons. The observed neural dysfunction is thought to be altered indirectly due to sequelae of SARS-CoV-2 infection of peripheral support cells by local inflammation that could compromise structural and functional features in including destruction of ciliary structure and dampened OSN firing.

Need for In Vitro Models to Study COVID-19 Anosmia: Given the emerging central role of olfactory support cells in COVID pathophysiology, an emerging area of research is focused around to find out how primary infection of non-neural cells (like the support cells) can alter the odour perception. Delineating the pathophysiological mechanisms underlying anosmia following SARS-CoV-2 infection requires interrogative experiments aimed at determining the mechanistic links between viral infection, sustentacular (olfactory epithelial supporting cells) cell dysfunction and altered OSN signalling. The anatomical proximity of sustentacular cell processes and OSN cilia predicts a direct role of supporting cells, but the nature of this support

and how it might be abrogated after SARS-CoV-2 infection remain unestablished. Therefore, in order to address mechanistic links of viral-mediated anosmia in COVID-19 pathology, the development of superior in vitro models where human ACE2 and TMPRSS2 can be made to express as opposed to viral counterparts and rigorous non-mouse model organisms, which are susceptible to infection by SARS-CoV-2, is encouraged (Bryche et al. 2020; Munster et al. 2020; Shi et al. 2020; Sia et al. 2020). Establishment of faithful models that can recapitulate triggering of viral immune pathways in vitro in readily accessible virus-permissive cell types would help understand the molecular players and determine potential therapeutic targets without the need of a BSL-3 containment system. Importantly, currently there are no rigorous in vitro models of olfactory epithelium housing the COVID-19 relevant supporting cells that can be used to study the pathobiology of SARS-CoV-2 infection and anosmia.

Current manifestation of diverse symptoms in COVID-19 patients makes it elusive to know whether COVID-19 attacks chemosensation through one or more pathophysiological mechanisms or whether specific smell or taste qualities are affected. Further, there is a lack of an understanding of how smell, taste and chemesthesis evolve over the long term in the subset of patients who have prolonged recovery times and how non-anosmia SARS-CoV-2-positive patients can afford to stay asymptomatic. Decoding these mechanisms by which SARS-CoV-2 influences chemical sensing will have important implications for our understanding of how viruses can functionally alter sensory systems and how anosmia-associated mechanistic pathways can be exploited to design better therapeutics and how related distinct/alternate escape mechanisms can predict better prognosis. Quantitative assessment of olfactory dysfunction has been reported to be detected accurately in asymptomatic COVID-19 carriers recently (Bhattacharjee et al. 2020), and novel in vivo virus-free animal models that involve dsRNA-triggered interferon immune signalling in the olfactory epithelium are employed for modelling transient smell loss in COVID-19 patients (Rodriguez et al. 2020).

However, virus-free in vitro models that can recapitulate olfactory epithelium-associated changes in odorant receptor expression, attendant cyclic AMP signalling pathways and altered olfactory sensory neuron firing are not reported till date, thereby emphasizing the need to establish faithful and tractable OE neurospheres/OE tissue-like models that recapitulate viable supporting cell and sensory neuron crosstalk in vitro. Such models can be leveraged to study the mechanistically driven chemosensory changes in symptomatic and asymptomatic COVID-19 patients and predict (a) early chemosensory changes (OR expression patterns and OSN electrophysiological patterns) and disease outcomes and (b) potential disease-spreading asymptomatic carriers. The mouse and human ACE2 and TMPRSS2 expression data align well in the olfactory epithelium (PNS) (Brann et al. 2020), but there are clinically relevant inter-species divergences in the olfactory bulb and brain (CNS). Importantly, ACE2 gene is regulated by inflammatory mediators in human cells, and it is speculated that other SARS-CoV-2 entry genes (like serine proteaseTMPRSS2) may be similarly modulated by primary infection and inflammation (Ziegler et al. 2020; Ansari et al. 2020).

Vision for Hearing and Olfaction (Innovative Regenerative Approaches to Treat ENT Pathologies): Establishment of cranial sensory placodes by employing novel cellular-stress stem cell reprogramming models with implications in studying sensorineural hearing loss regeneration and COVID-19-associated anosmia.

Our group is primarily interested to explore exciting, novel (simplistic), autologous, native/intrinsic and physiological approaches to trigger reprograming of adult cells, which can help overcome the inherent roadblocks and limitations in the current field of establishment of productive pluripotency signatures and successful differentiation into desirable cell types/tissue for prospective clinical applications. Interaction with the world around us requires extracting meaningful signals to guide behaviour. The mammalian senses of olfaction, vision, somatosensation, hearing, balance and taste facilitate extraction of sense-specific information. Most sensory organs in the vertebrate head originate from cranial placodes (CPs). CPs are formed *embryonically* through a series of differentiation steps arising at the boundary between neural and non-neural ectoderm, and they can be divided into anterior, posterior and intermediate groups depending on their place of origin in the developing embryonic head. Anterior CPs include adenohypophyseal, olfactory and lens placodes; intermediate CPs include the trigeminal placode, which gives rise to the sensory neurons of the ophthalmic and maxilla-mandibular divisions of the trigeminal ganglion; posterior CPs are comprised of the otic, lateral line placode and the epibranchial placodes that give rise to the inner ear, lateral line organs (in fish and amphibian) and sensory neurons of the geniculate, petrosal and nodose ganglia, respectively (Fig. 7.1).

Current Regenerative Approach for Hearing Loss: We are primarily interested in building the otic placodes that can form viable otic vesicles in vitro, which can be further directed to generate cochlear/vestibular systems of inner ear and the sensory neurons of its associated vestibulocochlear (VIIIth) ganglion (Fig. 7.1).

Current State of the Art: Directed differentiation protocols using human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) modulate developmental FGF, BMP, TGFβ and WNT signalling pathways to generate desired cell fates within the ectoderm. Interestingly, manipulating the culture conditions by addition of TGFβ antagonists and BMP4 agonists can skew pluripotent cells towards epidermal and placodal fates. Further, FGF signalling concomitant with attenuated BMP and WNT signalling triggers placodal emergence marked by expression of SIX1 and EYA1/2 and FGF signalling coupled to intermediate BMP signalling triggers neural crest formation (PAX3/7 and SOX9/10). Therefore, *FGF* signalling is central/integral to *placodal induction*.

Challenges in Placodal Induction: Some studies indicate that both NC (neural crest) and PPR (pre-placodal region) are derived from a common progenitor population arising in the neural border region. However, other studies suggest that PPR and NC arise from distinct ectodermal regions of varying competence, with NC arising from neural plate and PPR arising from non-neural ectoderm. Therefore, the complexity of neural plate border specification poses a major limitation to gain deeper mechanistic insights into the developmental cues driving efficient placodal differentiation; hence generation and establishment of in vitro models with improved cranial

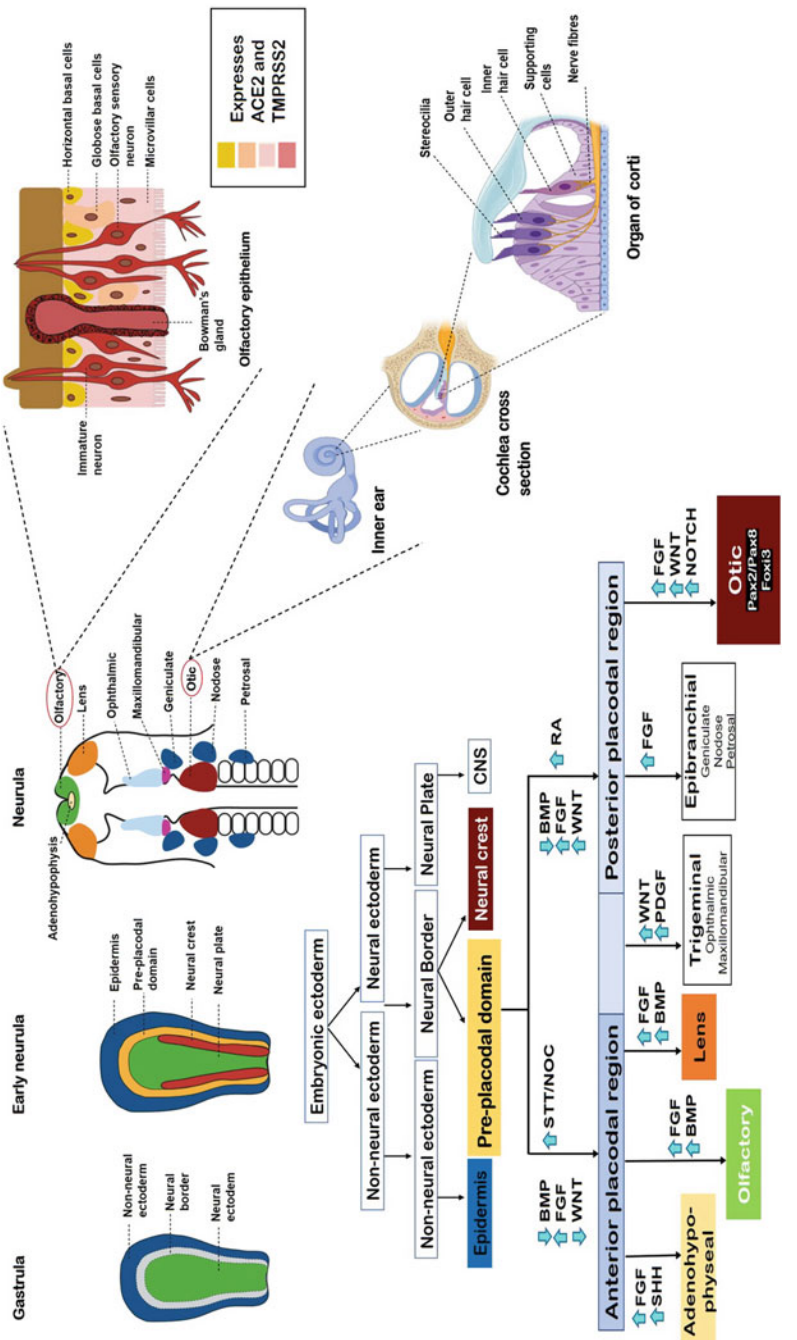


Fig. 7.1 Placode development begins with induction of non-neural ectoderm and neural ectoderm from embryonic ectoderm in vivo. Neural border forms in between neural and non-neural ectoderm. The neural border gives rise to pre-placodal domain and neural crest. Different cranial placodes arise from the pre-placodal domain under the influence of specific signals. The cellular models of otic progenitor sensory cells and inner ear organoids goal at achieving

placode differentiation are challenging. We established and developed an interesting in vitro 3D collagen gel-based cellular model for delineating epithelial-fibroblast crosstalk, which was marked by the secretion of FGF2/bFGF (Sharma et al. 2013). Fibroblast growth factor (FGF) signalling is regarded as indispensable step in the induction of otic placode/progenitors and inner ear organoids (Ealy et al. 2016; Koehler et al. 2017; Lenz et al. 2019; Lopez-Juarez et al. 2019; Roccio et al. 2018; Lahlou et al. 2018; Chen et al. 2018; Senn et al. 2020; Xia et al. 2019; McGovern et al. 2019; Roccio and Edge 2019; Ono et al. 2014; Ladher et al. 2005, 2010; Alvarez et al. 2003; Barald and Kelley 2004). Given that FGF signalling is central/integral to *placodal induction*, we are interested to harness the potential of this culture system to build placodes in a dish. We are, therefore, encouraged to establish viable in vitro model systems to develop an improved protocol for placodal differentiation to facilitate sensory regeneration and possibly benefit ongoing restoration attempts of impaired senses.

7.6 Precision Medicine for Genetic SNHL Treatment

We are working towards exploiting the translational potential of the genetic heterogeneity in deafness for future precision medicine-based treatment to SNHL patients. Though the extraordinary heterogeneity in HL confounds diagnosis, however, it also positions hearing specialists with a potential to offer effective individualized/customized stem cell/gene therapy treatments driven by unique patient-specific variant(s) for genetically deaf (Rudman et al. 2018), given the genetics has been well established. Towards this, we are working to develop molecular genetics of SNHL patients to be able to gene-edit the most pathogenic variant(s) using CRISPR editing tool in patient-specific disease models and evaluate the functional outcomes of the diseased and the corresponding gene-edited isogene controls in the established otic prosensory progenitor cells (OPSCs) and/or inner ear organoids (IEOs). The OPSCs and IEOs are generated in our lab by employing novel cellular stress models involving serine protease-based reprogramming methodology (Sharma et al. 2017, 2019), which is exploited to coax the reprogrammed cells to differentiate into inner ear organoid-like structures expressing inner ear-specific markers (Fig. 7.2a, b). We are further optimizing the establishment of patient-specific cell culture models that may facilitate patient-tailored treatment for SNHL.

Moreover, we propose to integrate our genetic and stem cell data into a viable translational approach for HL patients. In cases with predictive positive CI outcome,

Fig. 7.1 (continued) successful recreation of the organ of Corti cells including inner and outer hair cells, supporting cells and auditory neurons in vitro using stem cell reprogramming followed by differentiation of desirable cells by employing developmental morphogens and signalling cues. The COVID-19 pandemic-associated anosmia has encouraged the establishment of novel olfactory epithelial neurosphere culture models that can harbour disease-relevant cell types (supporting cells, stem cells and olfactory neurons) to delineate the cellular and molecular targets in anosmia

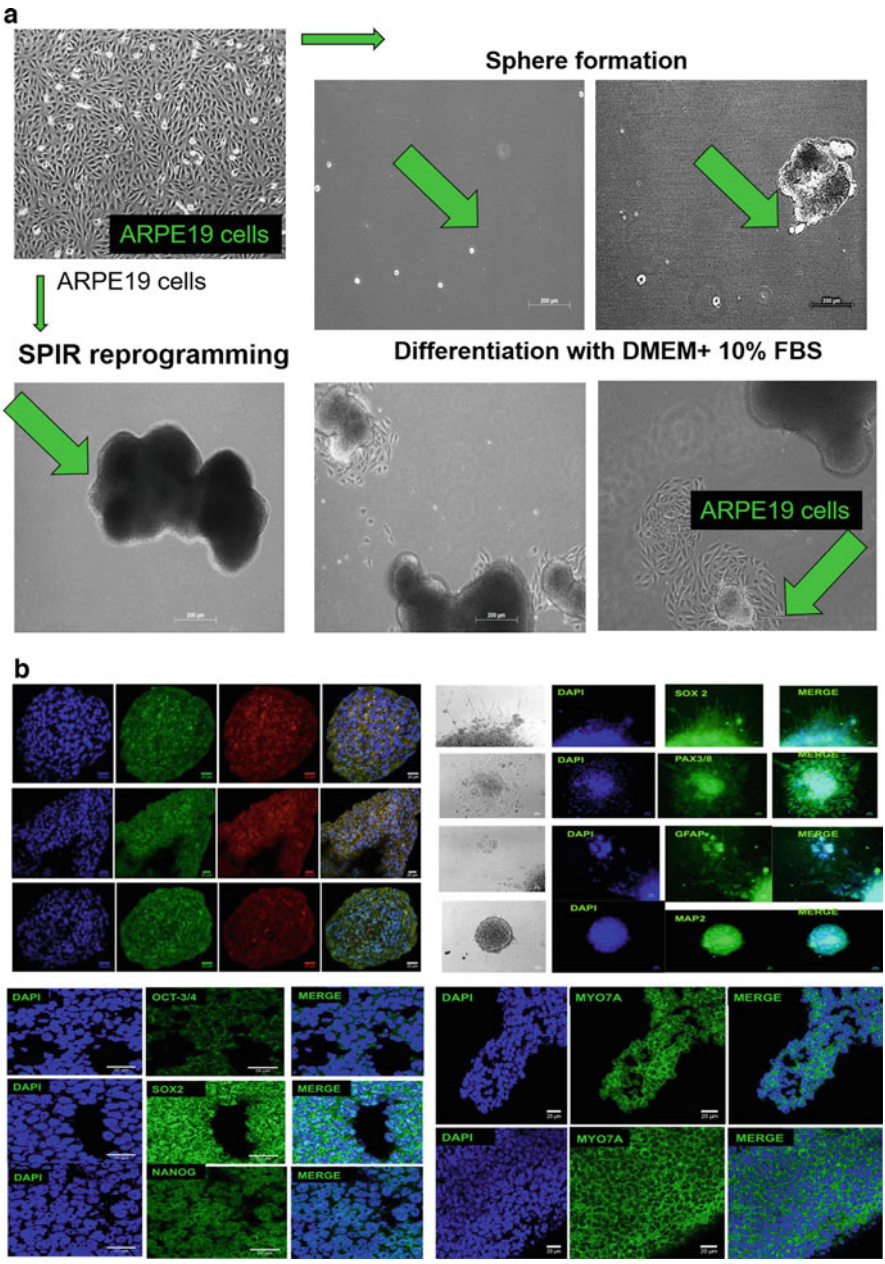


Fig. 7.2 (**a**) 3D spheroid formation with protease stress-induced reprogramming (SPIR) of neuroepithelial ARPE19 (adult retinal pigment epithelial) cell line (**b**) SPIR conditions-induced otic progenitor/associated (Nestin, Ecad, Beta-catenin) and inner ear organoid marker-supporting cells (SOX2), neuronal cells (MAP2, GFAP) and inner hair cell markers (PAX8, MYO7A)

the use of cochlear implant is expected to be expanded to serve as a potential carrier for delivering intracochlear drug, gene and stem cell therapies, as well as extracochlear and combination drug therapies (Duan et al. 2004; Eshraghi et al. 2007; Dinh et al. 2008; Jolly et al. 2010; Farhadi et al. 2013; Suckfuell et al. 2014; Plontke 2017). We hope to lay a foundational step and open avenues towards better hearing restoration by delivering combinatorial autologous stem cell therapy with cochlear implant in the Indian patients, in which a successful gene-phenotype correlation and positive CI outcome get established. There is also hope for delivering stem cells along with a repeated CI intervention in patients with a prior failed CI, which will be determined by successful identification and gene editing of the causative mutant/variant that earlier prevented CI stimulation (possible mutations in spiral ganglion/cochlear nerve). Establishing novel genotype-phenotype associations in Indian patients will contribute to diagnostics and also to (preclinical) the development of therapeutic strategies. The experimental evaluation of potential functional (pathogenic) effects of variants would be carried out in our novel cellular stress models involving serine protease-based reprogramming methodology that generates inner ear organoid-like structures expressing inner ear-specific markers (Fig. 7.2).

7.7 Novel Cellular Stress Model for Studying Molecular and Cellular Targets Underlying COVID-19-Related Anosmia

Given the copious proteolytic stress underlying COVID-19 pathogenesis, we are encouraged to leverage our proteolytic stress models (Sharma et al. 2021) and accepted manuscript (Sharma and Panda 2020) towards the establishment and characterization of novel olfactory epithelial (OE) neurospheres housing supporting cells, progenitor cells and sensory neurons (Fig. 7.1); the cytokine/inflammatory stress will be recapitulated by preconditioning these OE neurospheres in the presence of inflammatory mediator-rich plasma of SARS-CoV-2 patients with and without anosmia and further characterized for ACE2, TMPRSS2, OE-specific markers and odorant receptor marker expression by immunofluorescence. Transcriptomic profiling will be performed to investigate novel anosmia-associated deregulated pathways.

We are also interested to investigate the role of acute-phase protein A1AT (α/alpha 1 anti-trypsin) in the pathogenesis of COVID-19. Importantly, A1AT is the major physiological inhibitor of neutrophil elastase; moreover, our pilot findings strongly link the partial loss/downregulation of A1AT in our experimental setup to the biological pathways linked to productive viral infection, subversion of host antiviral interferon immune response and deregulation of thromboinflammatory mediators as recently reported in our preliminary findings (https://www.preprints.org/manuscript/202006.0206/v1) (Sharma and Panda 2020). Besides our correlative data on A1AT and dampened host response, recent emerging studies are increasingly implicating supplementation of exogenous A1AT as a novel therapy that may work

via modulating altered neutrophil metabolic networks in the treatment of COVID-19 disease (McElvaney et al. 2020), and A1AT has been increasingly linked to pathogenesis and therapeutics of COVID-19 (Oguntuyo et al. 2020) and is proposed as a host protective factor against SARS-CoV-2 (de Loyola et al. 2020; Shapira et al. 2020). Mechanistically, A1AT is proposed to inhibit SARS-CoV-2 infectivity by inhibiting the obligatory viral entry serine protease TMPRSS2, which in conjunction with proteases ACE2 and ADAM17 is predicted as main risk factor for COVID-19 (Zipeto et al. 2020).

7.8 Repurposing of A1AT in COVID-19: The Potential and Conviction

A1AT is at the intersection node of coagulation and innate immune system. Interestingly, a spontaneous mutant of A1AT, A1AT-P (M358R), acquires antithrombin as opposed anti-neutrophil elastase activity, thereby linking A1AT and its variants to "serine protease/anti-protease stoichiometric deregulation" underlying coagulopathies (such as COVID-19). Further, A1AT-P scaffold has been adapted to acquire functionality to inhibit furin and complement factor C1s to yield a Serp1 (viral serpin), inhibiting furin pro-convertase-mediated viral pathogenicity. Serpin PC (also called α1-AT KRK) and α1-AT-Fc fusion proteins are other designer SERPINs in early clinical trials (NCT04073498; NCT03815396, 2019). The crystallographic datasets for wild-type and mutant AAT proteins point to considerable variability in the surface clefts, indicative of AAT protein as a potential target for in silico structure-based drug designing to promote homeostasis rebalancing in coagulopathies involving thrombo-inflammation (De Maat et al. 2019; Schmaier 2019; Luo et al. 2020). We are very hopeful to have another SARS-CoV-2-specific viral SERPIN in immediate future.

7.9 Therapeutic Vaccine and Prophylactic Viral Nose Filter Prototypes

We would attempt developing a A1AT-based prototype nose filter in lab to evaluate the binding of oral/nasopharyngeal swab-sampled SARS-CoV-2 virus to 0.1-micron hydrophobic membrane filters impregnated/coated with lipid-interacting A1AT to develop prototype to guide developing future A1AT-based prophylactic inhalers, gels or nose filters to trap/inhibit the virus at the site of entry (nasal route) when the viral loads are low and manageable.

7.10 Therapeutic Delivery Approaches for Inner Ear Disorders and Infectious Conditions of Nose and Paranasal Sinuses

7.10.1 Round Window: The Gateway for Inner Ear Therapeutics

The greatest challenge in the management of sensorineural hearing loss is the precise delivery of therapeutic agents into the cochlea by overcoming the blood-labyrinth barrier while preserving the structural and functional component of the inner ear. Systemic toxicity and bioavailability of agent are the major roadblocks for the systemic therapy. Round window membrane (RWM) is a three-layered semipermeable membrane and is the only non-bony connection between the middle ear to the inner ear and has been extensively investigated as an effective route for administration of inner ear therapeutics in sensorineural hearing loss (Lalwani and McGuire 2005; Goycoolea and Lundman 1997). The bioavailability of the therapeutic agent into the inner ear can be accelerated by increasing the duration of stay of the reagent in the middle ear, increasing the RWM permeability and by direct intracochlear delivery (Swan et al. 2008; Plontke et al. 2016). The potential for each modality is the current trend in exploring the drug delivery to the inner ear treatment.

7.10.2 Transtympanic Modality

Injecting the agent in the middle ear is the simplest approach to deliver the drug into the inner ear. However, the duration of stay in the middle ear is the limiting factor for effective transport via the RWM. Rapid clearance of the agent through the Eustachian tube and the permeability of RWM are key variables for its bioavailability to inner ear via transtympanic route. Silverstein Microwick® and RW microcatheter with pump are two devices explored for delivering the drug to the RW by minimizing the spillage through the Eustachian tube (Silverstein et al. 2004; Pararas et al. 2012). However, both the devices require surgical procedure to enter the middle ear hence being more invasive. For more effective and less invasive procedure, the nature of the injectable solution has been studied to overcome the duration of stay. Hydrogels are recently studied carrier solutions which can be safely injected via transtympanic route (Mäder et al. 2018). These solutions change their viscosity on environmental triggers. Increased viscosity reduces the Eustachian tube clearance and consequently enhances the contact time of the agent with the RWM. One such hydrogel is Poloxamer 407 which is an injectable liquid at room temperature, but once injected it converts into gel form after coming in contact with the middle ear at body temperature allowing improved exposure time with RWM (Wang et al. 2009). Hyaluronic acid is a non-toxic biodegradable viscous non-sulphate glycosaminoglycan polysaccharide and has been used as a substrate to deliver therapeutic agent into the RWM (Selivanova et al. 2003). It is hypothesized that by changing the osmotic pressure across the RWM, hyaluronic acid enhances the RWM permeability (Bjurström et al. 1987). Recently nanoparticles have been explored in otological procedures for safe and effective delivery of therapeutics

across the RWM. Liposomes, hyperbranched polylysine (HBPL), lipid nanocapsule (LNC), superparamagnetic iron oxide nanoparticles (SPIONs), and poly lactic-co-glycolic acid (PLGA) are some well-studied nanocarriers (Pritz et al. 2013; Bozzuto and Molinari 2015; Ge et al. 2007). SPIONs have a magnetic core and can be magnetically controlled for safe delivery of agent to the inner ear. With continued research combination of different nanoparticles, hydrogels can be engineered for safe delivery of drug payload to the inner ear.

7.10.3 Intracochlear Therapy

Intracochlear therapy entails the direct delivery of therapeutic agent into the inner ear by passing the blood-labyrinth barrier and the RWM permeability issues. It is a surgical procedure where a standard cochleostomy/round window cochleostomy is required for accessing the cochlea and delivery of the therapeutic agent. The inherent issue with this route is the possibility of leak at the injection site (Plontke et al. 2016). Cochlear implantation is one of the practical solutions for effective intracochlear drug administration (Borenstein 2011). Biohybrid cochlear implants are coated implants for neurosensory restoration of the cochlea and have been studied in animal models and also in few human clinical trials (Roemer et al. 2016).

7.11 Cellular Models of Nasal Epithelium for Enhanced Transnasal Delivery of Drugs: An Overview

Nasal drug delivery has gained the focus and interest of the scientific community in the last two decades as an area of research due to the unique nature of the respiratory epithelium in the nasal cavity and paranasal sinuses. Conventionally nasal delivery of drugs is being used for allergic, infectious conditions of nose and paranasal sinuses. However, the concept of nasal drug delivery targeting a systemic action has gained momentum due to features of high surface area for increased bioavailability, high blood flow and bypass of the hepatic first pass effect (Illum 2002; Leonard et al. 2007), especially for the drugs susceptible to enzymatic degradation (Illum 2003; Mygind and Dahl 1998). Though the concept of intranasal drug arose back in the 1980s (Chang and Chien 1984), the major focus developed in the initial years of twenty-first century (Lipworth and Jackson 2000). Development of various cellular models for the study of transnasal drug delivery is a primary requirement for exploring this possibility.

7.11.1 Nasal Epithelium as a Functional Unit for Drug Delivery

Nasal epithelium contains ciliated, non-ciliated, columnar, goblet, basal and few neurosecretory cells and covers an area of 140–160 cm^2 which comprises of vestibule (1%), olfactory region (8–10%) and rest of the respiratory nasal and

paranasal epithelium (Baroody 2007) with turbinates and processes guiding the air flow to enhance contact of nasal epithelium with the breathed air (Morrison and Costanzo 1990). The mucosa is covered by a continuously replacing (every 15–20 min) mucous gel layer which plays a significant role in entrapment of substances and consequent action as either absorption or mucociliary clearance for administered or inhaled molecules along with its other significant role of humidification of air to assist breathing (Merkus et al. 1998).

7.11.2 Nasal Drug Delivery: Possibility and Concerns

There exists various factors which determine and affect nasal absorption of drugs and molecules through the epithelium such as variations in mucociliary clearance[4] (normal rate 5 mm/min) and resultant contact time of a substance to mucosa (Schipper et al. 1991); local enzymatic degradation by enzymes such as peptidases (Chung and Donovan 1996; Peterson et al. 2019); various pathophysiological conditions rhinitis/rhinosinusitis; various chemical and physical properties of the administered molecules (Costantino et al. 2007) such as molecular weight (significant decrease for MW $>$ 1000 Da), solubility, dissolution rate, charge, partition coefficient, particle size, presence of polymorphism, pH of the solution in which drug is dissolved, ionized fraction of the molecule (ionized fraction $1/\alpha$ absorption rate) and solute lipophilicity (lipophilicity α absorption rate); various chemical characters of formulation (dose, volume, pH, osmolarity, viscosity); physical form of drug (spray, drop, powder, cream); and mode of administration. The various studies have figured out two primary pathways of transnasal absorption of drug including a transcellular lipoidal pathway (Kandimalla and Donovan 2005; Chemuturi et al. 2006) (passive diffusion, carrier mediates and endocytosis/transcytosis) and paracellular/intercellular aqueous pathway (Johnson and Quay 2005; Johnson et al. 2008). The lipid-soluble drugs are primarily absorbed through the transcellular pathway in contrast to the aqueous soluble drugs absorbed primarily through the paracellular pathway. The paracellular pathway is affected by the proteins present in the tight junctions, including occludin, claudin isoforms, perijunctional actin making active change in the transcellular pore size and junctional adhesion molecules (JAM), and also by the substances present in drug that can modulate the size of the pores such as cholera toxin, polymer-based microspheres and tight junction-modulating peptides such as PN 159.

7.11.3 Studying Nasal Respiratory Epithelium Pathophysiology: Feasibility and Hindrances

Obtaining the nasal mucosal epithelium and maintenance of the exact conditions simulating the nasal mucosa, mucous cover and types of junctions along with the cellular and ciliary integrity in the in vitro excised mucosal models has been a difficult endeavour. This has led researchers to think of alternative in vivo animal

models and in vitro cellular models for studying and developing transnasal delivery systems and drugs which can ease the treatment process of many diseases. In vivo models have been tried in various animals (Chen et al. 2006; Wang et al. 2006) and also in in vitro animal cell line models as can be seen in a porcine cell model by Ladel et al. (Ladel et al. 2019). However, inter-species differences in the mucosal architecture and chemical and enzymatic nature have made drawing clear conclusions of the findings difficult. Hence, prior extracted and subsequently cultured nasal respiratory mucosal cells have emerged as feasible surrogate models for studying transnasal drug delivery as these cells demonstrate characters such as various transporters for transcellular absorption, tight junctions for paracellular absorption, mucin secretion and cilia for mucociliary clearance studies. The physiochemical condition and the type of culture media affect the integrity of these models (Yoo et al. 2003).

7.11.4 Cellular Models for Nasal Drug Delivery Studies

The initial cell line models to study the nasal mucosal physiology and metabolism include RPMI 2650 taken from tumour cells (Werner and Kissel 1996). This has a distinctive concern of forming multiple layers contrast to a confluent monolayer as seen in in vivo conditions; however, restrictive models with RPMI 2650 with efforts to produce epithelium have shown moderate results later (Sun et al. 2020; Bai et al. 2008). Hence, this is not clearly suitable for studying the transnasal transport mechanisms in spite of the presence of peri-junctional actin in these cells. This has led to the concept of in vitro cell culture models. Werner (Werner and Kissel 1995) reported the first ever primary human nasal epithelial cell model for transnasal absorption and transportation studies on Transwell^{R} insert, which has been used primarily to study various peptide transport including insulin. Difficulty in obtaining functional usable nasal mucosal cells, contamination and huge variations from donor to donor are considered primary hindrances for the widespread use of this model. Even with the development of this model, the question of creating functional tight junctions was a concern as this is an essential structure for studying transport and drug delivery mechanisms. Yoo (Yoo et al. 2003) used liquid-covered culture (LCC) method to solve this concern which led to formation of functional junctions with high transepithelial electrical resistance (up to 3000-ohm cm^2) which can be used for transport studies up to passage 4 cultures, though formation of a functional mucosal layer with ciliary integrity was still a concern. However, this model proved certain important findings that for aqueous paracellular pathways, the transcellular electric resistance and transcellular permeability were deciding factors and the permeability coefficient (P^{per}) varies accordingly. However, for the lipid-soluble drugs and peptides, the lipophilicity index (log P) was of significant importance to decide the P^{per} given the basic conditions of transepithelial electric gradient of at least 500 ohm cm^2 existing at the junctions (Yoo et al. 2003; Lin et al. 2005).

The development of functional mucous layer and cilia to emulate nasal epithelial conditions led to further research, and it was later discovered that using an

air-interfaced model (Lee et al. 2005) with exposure of the apical surface of the cells with air (from third day of seeding) instead of liquid covers and supply of culture media from basolateral sides with the addition of retinoic acid additives in a serum-less and hormone-supplemented environment led to better expression of functional mucosal layer with enhanced ciliary integrity and function along with maintenance of transepithelial electric resistance at the tight junctions with initial peak on day 5 in comparison to liquid-covered cultures in which the electric resistance reduced to near zero by day 15 suggesting better mucous layer formation, ciliary integrity as documented in electron microscopy, tight junction functionality and electric resistance integrity with air-interfaced cultures compared to the liquid-covered cultures, which makes air-interfaced cultures in Transwell^{R} insert better suitable for the transnasal transportation and drug delivery studies. The liquid-covered cultures used the Dulbecco's Modified Eagle Medium (DMEM) which has a significant role in cell proliferation due to the presence of 10% FBS. However the air-interfaced cultures required bronchial epithelial growth medium (BEGM) with supplemented retinoic acid and hormones in a serum-free environment (Gruenert et al. 1990; Mattinger et al. 2002) for better differentiation of mucous layer and ensuring ciliary development and integrity (Tang et al. 2017). Hence, a mixture of both mediums (Kim 2008) was found best for the air-interfaced cultures of nasal epithelial cells for drug delivery studies. Further development of these primary culture methods has also been discovered to simulate various physiological and pathological scenarios for studying mucosal function in various real-life situations. Xiaofang Wu (Wu et al. 2015) showed that human nasal epithelial basal cells (HNEBCs) cultured on Matrigel form glandular acini-like structures, and HNEBCs embedded in a collagen type I (Col I) matrix form a network of tubules to simulate real-life situations of glandular function in nasal mucosa. Kai Sen Tan (Tan et al. 2018) used rhinovirus-infected human nasal epithelial cells (HNEC) to produce CXCL9, IP-10, CXCL11 and RANTES as likely initiators of airway inflammatory responses in order to simulate viral rhinitis-like conditions. Tengfei Tian et al. used H3N2 influenza virus infection in order to increase Oncostatin M expression which has a significant role in tight junction alteration generating high permeability (Tian et al. 2018). Similarly, PAR-2 agonist SLIGRL-NH2 and antagonist FSLLRY-NH2 in the environment led to the modulation of PAR-2 expression in tight junctions, an increase in which led to the downregulation of zonula occludens (ZO-1) and claudin-1 leading to epithelial barrier dysfunction in allergic rhinosinusitis (Wang et al. 2020c). Bernadett Boda (Boda et al. 2018) used MucilAir™ (Huang et al. 2011), a human standardized air-liquid interface 3D airway epithelial culture, to study human nasal epithelial alterations in various viral rhinitis and could show that rhinovirus B14, C15 and influenza A(H1N1) induce significant increase of β-defensins 2 and cathelicidin release which can be modulated with specific antiviral drugs like oseltamivir. SmallAir™ is also another such respiratory epithelial system for studying function in various physiological and pathophysiological conditions (Huang et al. 2017). All these further developments are both liquid and air medium and air-interfaced models compared to the initial liquid cover culture cellular models.

Cellular models have come a long way from the initial days with concepts through excised cellular models, in vivo animal models, tumour cell line models to current-day primary human nasal epithelial cell line models. This has made drug delivery studies easy and a possibility for developing promising transnasal drug delivery by passing the enteral and the invasive parenteral routes. As of now azelastine, beclometasone, budesonide, levocabastine, mometasone, Fluticasone, olopatadine, sodium chromoglycate, triamcinolone acetonide and mupirocin are available as local medications with primary local and some systemic effects and similarly estradiol, nicotine, cyanocobalamin, desmopressin, oxytocin, salmon calcitonin, buserelin, nafarelin, zolmitriptan, sumatriptan, naloxone, fentanyl, butorphanol, insulin and live attenuated influenza vaccine are available for primary targeted systemic used through a transnasal delivery (Tucker et al. 2018; Pires et al. 2009). However, there is still a long way to go in terms of the studies on these cellular models where we can come with possible formulations of a number of drugs which can be administered in a transnasal route in addition to the few existing now. Cellular models form a primary basis for the future studies and remind humans of the achievements that can transform quality of lives in the future.

Acknowledgements The research work discussed above is conducted in the Department of Otolaryngology and Head and Neck Surgery under the following funding schemes: Intramural PGIMER grant No.71/2-Edu-16/1098, SERB extramural fundings SERBCRG/2019/006745 and SPR/2019/001447. We thank the funding agencies for the financial support and SERBCRG/2019/006745 for providing Junior Research Fellowship to Ms. Kavita Kaushal.

Conflict of Interest None.

References

Agyeman AA, Chin KL, Landersdorfer CB, Liew D, Ofori-Asenso R (2020) Smell and taste dysfunction in patients with COVID-19: a systematic review and meta-analysis. Mayo Clin Proc 95(8):1621–1631. Elsevier

Alvarez Y, Alonso MT, Vendrell V, Zelarayan LC, Chamero P, Theil T, Bösl MR, Kato S, Maconochie M, Riethmacher D, Schimmang T (2003) Requirements for FGF3 and FGF10 during inner ear formation. Development 130(25):6329–6338

Ansari MA, Marchi E, Ramamurthy N, Aschenbrenner D, Hackstein CP, Bowden R, Sharma E, Pedergnana V, Venkateswaran S, Kugathasan S, Mo A (2020) A gene locus that controls expression of ACE2 in virus infection. medRxiv

Arunachalam RK, Koshy T, Venkatesan V, Dawson GP, Franklin Durairaj Paul S, George P (2020) Mutation analysis using multiplex ligation-dependent probe amplification in consanguineous families in South India with a child with profound hearing impairment. Lab Med 51(1):56–65

Azaiez H, Booth KT, Bu F, Huygen P, Shibata SB, Shearer AE, Kolbe D, Meyer N, Black-Ziegelbein EA, Smith RJ (2014) TBC 1 D 24 mutation causes autosomal-dominant nonsyndromic hearing loss. Hum Mutat 35(7):819–823

Azaiez H, Decker AR, Booth KT, Simpson AC, Shearer AE, Huygen PL, Bu F, Hildebrand MS, Ranum PT, Shibata SB, Turner A (2015) HOMER2, a stereociliary scaffolding protein, is essential for normal hearing in humans and mice. PLoS Genet 11(3):e1005137

Azaiez H, Booth KT, Ephraim SS, Crone B, Black-Ziegelbein EA, Marini RJ, Shearer AE, Sloan-Heggen CM, Kolbe D, Casavant T, Schnieders MJ (2018) Genomic landscape and mutational signatures of deafness-associated genes. Am J Hum Genet 103(4):484–497

Bai S, Yang T, Abbruscato TJ, Ahsan F (2008) Evaluation of human nasal RPMI 2650 cells grown at an air-liquid interface as a model for nasal drug transport studies. J Pharm Sci 97(3): 1165–1178

Barald KF, Kelley MW (2004) From placode to polarization: new tunes in inner ear development. Development 131(17):4119–4130

Bardhan T, Jeng JY, Waldmann M, Ceriani F, Johnson SL, Olt J, Rüttiger L, Marcotti W, Holley MC (2019) Gata3 is required for the functional maturation of inner hair cells and their innervation in the mouse cochlea. J Physiol 597(13):3389–3406

Barnes BJ, Adrover JM, Baxter-Stoltzfus A, Borczuk A, Cools-Lartigue J, Crawford JM, Daßler-Plenker J, Guerci P, Huynh C, Knight JS, Loda M (2020) Targeting potential drivers of COVID-19: neutrophil extracellular traps. J Exp Med 217(6)

Baroody FM (2007) Nasal and paranasal sinus anatomy and physiology. Clin Allergy Immunol 19: 1–21

Bauer PW, Geers AE, Brenner C, Moog JS, H. Smith RJ. (2003) The effect of GJB2 allele variants on performance after cochlear implantation. Laryngoscope 113(12):2135–2140

Bénézit F, Le Turnier P, Declerck C, Paillé C, Revest M, Dubée V, Tattevin P, Arvieux C, Baldeyrou M, Chapplain JM, Comacle P (2020) Utility of hyposmia and hypogeusia for the diagnosis of COVID-19. Lancet Infect Dis

Bhalla S, Sharma R, Khandelwal G, Panda NK, Khullar M (2009) Low incidence of GJB2, GJB6 and mitochondrial DNA mutations in North Indian patients with non-syndromic hearing impairment. Biochem Biophys Res Commun 385(3):445–448

Bhalla S, Sharma R, Khandelwal G, Panda NK, Khullar M (2011) Absence of GJB6 mutations in Indian patients with non-syndromic hearing loss. Int J Pediatr Otorhinolaryngol 75(3):356–359

Bhattacharjee AS, Joshi SV, Naik S, Sangle S, Abraham NM (2020) Quantitative assessment of olfactory dysfunction accurately detects asymptomatic COVID-19 carriers. EClinicalMedicine 16:100575

Bilinska K, Butowt R (2020) Anosmia in COVID-19: a bumpy road to establishing a cellular mechanism. ACS Chem Neurosci 11(15):2152–2155

Bjurström S, Slepecky N, Angelborg C (1987) A histopathological study of the inner ear after the administration of hyaluronan into the middle ear of the guinea pig. Acta Otolaryngol 104(Suppl 442):62–65

Blanco-Sánchez B, Clément A, Fierro J Jr, Stednitz S, Phillips JB, Wegner J, Panlilio JM, Peirce JL, Washbourne P, Westerfield M (2018) Grxcr1 promotes hair bundle development by destabilizing the physical interaction between harmonin and Sans Usher syndrome proteins. Cell Rep 25(5):1281–1291

Bock JO, Ortea I (2020) Re-analysis of SARS-CoV-2-infected host cell proteomics time-course data by impact pathway analysis and network analysis: a potential link with inflammatory response. Aging (Albany NY) 12(12):11277

Boda B, Benaoudia S, Huang S et al (2018) Antiviral drug screening by assessing epithelial functions and innate immune responses in human 3D airway epithelium model. Antivir Res 156:72–79

Bojkova D, Klann K, Koch B, Widera M, Krause D, Ciesek S, Cinatl J, Münch C (2020) Proteomics of SARS-CoV-2-infected host cells reveals therapy targets. Nature 583(7816): 469–472

Booth KT, Azaiez H, Kahrizi K, Simpson AC, Tollefson WT, Sloan CM, Meyer NC, Babanejad M, Ardalani F, Arzhangi S, Schnieders MJ (2015) PDZD7 and hearing loss: more than just a modifier. Am J Med Genet A 167(12):2957–2965

Booth KT, Azaiez H, Kahrizi K, Wang D, Zhang Y, Frees K, Nishimura C, Najmabadi H, Smith RJ (2018) Exonic mutations and exon skipping: lessons learned from DFNA5. Hum Mutat 39(3): 433–440

Borenstein JT (2011) Intracochlear drug delivery systems. Expert Opin Drug Deliv 8(9):1161–1174
Boulet SL, Boyle CA, Schieve LA (2009) Health care use and health and functional impact of developmental disabilities among US children, 1997-2005. Arch Pediatr Adolesc Med 163(1): 19–26
Bozzuto G, Molinari A (2015) Liposomes as nanomedical devices. Int J Nanomedicine 10:975
Brann DH, Tsukahara T, Weinreb C, Lipovsek M, Van den Berge K, Gong B, Chance R, Macaulay IC, Chou HJ, Fletcher RB, Das D (2020) Non-neuronal expression of SARS-CoV-2 entry genes in the olfaory system suggests mechanisms underlying COVID-19-associated anosmia. Sci Adv eabc5801
Bryche B, St Albin A, Murri S, Lacôte S, Pulido C, Gouilh MA, Lesellier S, Servat A, Wasniewski M, Picard-Meyer E, Monchatre-Leroy E (2020) Massive transient damage of the olfactory epithelium associated with infection of sustentacular cells by SARS-CoV-2 in golden Syrian hamsters. Brain Behav Immun 89:579–586
Butowt R, Bilinska K, Von Bartheld CS (2020a) Chemosensory dysfunction in COVID-19: integration of genetic and epidemiological data points to D614G spike protein variant as a contributing factor. ACS Chem Neurosci 11(20):3180–3184
Butowt R, Pyrc K, von Bartheld CS (2020b) Battle at the entrance gate: CIITA as a weapon to prevent the internalization of SARS-CoV-2 and Ebola viruses. Signal Transduct Targeted Ther 5(1):1–2
Cantuti-Castelvetri L, Ojha R, Pedro LD, Djannatian M, Franz J, Kuivanen S, van der Meer F, Kallio K, Kaya T, Anastasina M, Smura T (2020) Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. Science 370(6518):856–860
Canzi P, Pecci A, Manfrin M, Rebecchi E, Zaninetti C, Bozzi V, Benazzo M (2016) Severe to profound deafness may be associated with MYH9-related disease: report of 4 patients. Acta Otorhinolaryngol Ital 36(5):415
Carpena NT, Lee MY (2018) Genetic hearing loss and gene therapy. Genomics Informatics 16(4)
Chacko LJ, Sergi C, Eberharter T, Dudas J, Rask-Andersen H, Hoermann R, Fritsch H, Fischer N, Glueckert R, Schrott-Fischer A (2020) Early appearance of key transcription factors influence the spatiotemporal development of the human inner ear. Cell Tissue Res 379(3):459–471
Chang S, Chien Y (1984) Intranasal drug administration for systemic medication. Pharm Int 5(12): 287–288
Chemuturi NV, Haraldsson JE, Prisinzano T, Donovan M (2006) Role of dopamine transporter (DAT) in dopamine transport across the nasal mucosa. Life Sci 79(14):1391–1398
Chen SC, Eiting K, Cui K et al (2006) Therapeutic utility of a novel tight junction modulating peptide for enhancing intranasal drug delivery. J Pharm Sci 95(6):1364–1371
Chen W, Jongkamonwiwat N, Abbas L, Eshtan SJ, Johnson SL, Kuhn S, Milo M, Thurlow JK, Andrews PW, Marcotti W, Moore HD (2012) Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. Nature 490(7419):278–282
Chen JR, Tang ZH, Zheng J, Shi HS, Ding J, Qian XD, Zhang C, Chen JL, Wang CC, Li L, Chen JZ (2016) Effects of genetic correction on the differentiation of hair cell-like cells from iPSCs with MYO15A mutation. Cell Death Differ 23(8):1347–1357
Chen J, Hong F, Zhang C, Li L, Wang C, Shi H, Fu Y, Wang J (2018) Differentiation and transplantation of human induced pluripotent stem cell-derived otic epithelial progenitors in mouse cochlea. Stem Cell Res Ther 9(1):230
Chen J, Jiang Q, Xia X, Liu K, Yu Z, Tao W, Gong W, Han JD (2020) Individual variation of the SARS-CoV-2 receptor ACE2 gene expression and regulation. Aging Cell 19(7):e13168
Chung FY, Donovan MD (1996) Nasal pre-systemic metabolism of peptide drugs: substance P metabolism in the sheep nasal cavity. Int J Pharm 128(1-2):229–237
Contrera KJ, Betz J, Li L, Blake CR, Sung YK, Choi JS, Lin FR (2016) Quality of life after intervention with a cochlear implant or hearing aid. Laryngoscope 126(9):2110–2115
Cooper KW, Brann DH, Farruggia MC, Bhutani S, Pellegrino R, Tsukahara T, Weinreb C, Joseph PV, Larson ED, Parma V, Albers MW (2020) COVID-19 and the chemical senses: supporting players take center stage. Neuron 107(2):219–233

Corns LF, Johnson SL, Roberts T, Ranatunga KM, Hendry A, Ceriani F, Safieddine S, Steel KP, Forge A, Petit C, Furness DN (2018) Mechanotransduction is required for establishing and maintaining mature inner hair cells and regulating efferent innervation. Nat Commun 9(1):1–5

Cosh S, Helmer C, Delcourt C, Robins TG, Tully PJ (2019) Depression in elderly patients with hearing loss: current perspectives. Clin Interv Aging 14:1471

Costantino HR, Illum L, Brandt G, Johnson PH, Quay SC (2007) Intranasal delivery: physicochemical and therapeutic aspects. Int J Pharm 337(1–2):1–24

Cullen RD, Buchman CA, Brown CJ, Copeland BJ, Zdanski C, Pillsbury HC III, Shores CG (2004) Cochlear implantation for children with GJB2-related deafness. Laryngoscope 114(8): 1415–1419

Cunningham CL, Müller U (2019) Molecular structure of the hair cell mechanoelectrical transduction complex. Cold Spring Harb Perspect Med 9(5):a033167

Curhan SG, Willett WC, Grodstein F, Curhan GC (2020) Longitudinal study of self-reported hearing loss and subjective cognitive function decline in women. Alzheimers Dement 16(4): 610–620

Czajkowski A, Mounier A, Delacroix L, Malgrange B (2019) Pluripotent stem cell-derived cochlear cells: a challenge in constant progress. Cell Mol Life Sci 76(4):627–635

Davcheva-Chakar M, Sukarova-Stefanovska E, Ivanovska V, Lazarevska V, Filipche I, Zafirovska B (2014) Speech perception outcomes after cochlear implantation in children with GJB2/DFNB1 associated deafness. Balkan Med J 31(1):60

de Loyola MB, Dos Reis TT, de Oliveira GX, da Fonseca Palmeira J, Argañaraz GA, Argañaraz ER (2020) Alpha-1-antitrypsin: a possible host protective factor against Covid-19. Rev Med Virol 26:e2157

De Maat S, Sanrattana W, Mailer RK, Parr NM, Hessing M, Koetsier RM, Meijers J, Pasterkamp G, Renné T, Maas C (2019) Design and characterization of α1-antitrypsin variants for treatment of contact system–driven thromboinflammation. Blood 134(19):1658–1669

Defourny J (2019) Eph/ephrin signalling in the development and function of the mammalian cochlea. Dev Biol 449(1):35–40

Ding D, Liu H, Qi W, Jiang H, Li Y, Wu X, Sun H, Gross K, Salvi R (2016) Ototoxic effects and mechanisms of loop diuretics. J Otol 11(4):145–156

Dinh C, Hoang K, Haake S, Chen S, Angeli S, Nong E, Eshraghi AA, Balkany TJ, Van De Water TR (2008) Biopolymer-released dexamethasone prevents tumor necrosis factor α-induced loss of auditory hair cells in vitro: implications toward the development of a drug-eluting cochlear implant electrode array. Otol Neurotol 29(7):1012–1019

Duan M, Venail F, Spencer N, Mezzina M (2004) Treatment of peripheral sensorineural hearing loss: gene therapy. Gene Ther 11(1):S51–S56

Dunbar LA, Patni P, Aguilar C, Mburu P, Corns L, Wells HR, Delmaghani S, Parker A, Johnson S, Williams D, Esapa CT (2019) Clarin-2 is essential for hearing by maintaining stereocilia integrity and function. EMBO Mol Med 11(9):e10288

Duran Alonso MB, Lopez Hernandez I, de la Fuente MA, Garcia-Sancho J, Giraldez F, Schimmang T (2018) Transcription factor induced conversion of human fibroblasts towards the hair cell lineage. PLoS One 13(7):e0200210

Dyballa S, Savy T, Germann P, Mikula K, Remesikova M, Špir R, Zecca A, Peyriéras N, Pujades C (2017) Distribution of neurosensory progenitor pools during inner ear morphogenesis unveiled by cell lineage reconstruction. elife 6:e22268

Ealy M, Ellwanger DC, Kosaric N, Stapper AP, Heller S (2016) Single-cell analysis delineates a trajectory toward the human early otic lineage. Proc Natl Acad Sci 113(30):8508–8513

Ebrahim S, Ingham NJ, Lewis MA, Rogers MJ, Cui R, Kachar B, Pass JC, Steel KP (2016) Alternative splice forms influence functions of whirlin in mechanosensory hair cell stereocilia. Cell Rep 15(5):935–943

Eisenberger T, Di Donato N, Decker C, Delle Vedove A, Neuhaus C, Nürnberg G, Toliat M, Nürnberg P, Mürbe D, Bolz HJ (2018) A C-terminal nonsense mutation links PTPRQ with autosomal-dominant hearing loss, DFNA73. Genet Med 20(6):614–621

Elbracht M, Senderek J, Eggermann T, Thürmer C, Park J, Westhofen M, Zerres K (2007) Autosomal recessive postlingual hearing loss (DFNB8): compound heterozygosity for two novel TMPRSS3 mutations in German siblings. J Med Genet 44(6):e81

Eliezer M, Hautefort C, Hamel AL, Verillaud B, Herman P, Houdart E, Eloit C (2020) Sudden and complete olfactory loss function as a possible symptom of COVID-19. JAMA Otolaryngol Head Neck Surg

Elliott KL, Fritzsch B, Duncan JS (2018) Evolutionary and developmental biology provide insights into the regeneration of organ of corti hair cells. Front Cell Neurosci 12:252

Ellwanger DC, Scheibinger M, Dumont RA, Barr-Gillespie PG, Heller S (2018) Transcriptional dynamics of hair-bundle morphogenesis revealed with CellTrails. Cell Rep 23(10):2901–2914

Eshraghi AA, Adil E, He J, Graves R, Balkany TJ, Van De Water TR (2007) Local dexamethasone therapy conserves hearing in an animal model of electrode insertion trauma-induced hearing loss. Otol Neurotol 28(6):842–849

Farhadi M, Jalessi M, Salehian P, Ghavi FF, Emamjomeh H, Mirzadeh H, Imani M, Jolly C (2013) Dexamethasone eluting cochlear implant: histological study in animal model. Cochlear Implants Int 14(1):45–50

Fedick AM, Jalas C, Swaroop A, Smouha EE, Webb BD (2016) Identification of a novel pathogenic OTOF variant causative of nonsyndromic hearing loss with high frequency in the Ashkenazi Jewish population. Appl Clin Genet 9:141

Fettiplace R, Nam JH (2019) Tonotopy in calcium homeostasis and vulnerability of cochlear hair cells. Hear Res 376:11–21

Fodoulian L, Tuberosa J, Rossier D, Landis B, Carleton A, Rodriguez I (2020) SARS-CoV-2 receptor and entry genes are expressed by sustentacular cells in the human olfactory neuroepithelium. BioRxiv

Fontanet A, Grant R, Tondeur L, Madec Y, Grzelak L, Cailleau I, Ungeheuer MN, Renaudat C, Pellerin SF, Kuhmel L, Staropoli I (2020) SARS-CoV-2 infection in primary schools in northern France: a retrospective cohort study in an area of high transmission. MedRxiv

Forge A, Schacht J (2000) Aminoglycoside antibiotics. Audiol Neurotol 5(1):3–22

Fox SE, Akmatbekov A, Harbert JL, Li G, Brown JQ, Vander Heide RS (2020) Pulmonary and cardiac pathology in Covid-19: the first autopsy series from New Orleans. MedRxiv

Fritzsch B, Pan N, Jahan I, Elliott KL (2015) Inner ear development: building a spiral ganglion and an organ of Corti out of unspecified ectoderm. Cell Tissue Res 361(1):7–24

Fritzsch B, Elliott KL, Pavlinkova G (2019) Primary sensory map formations reflect unique needs and molecular cues specific to each sensory system. F1000Res 8

Fukunaga I, Fujimoto A, Hatakeyama K, Aoki T, Nishikawa A, Noda T, Minowa O, Kurebayashi N, Ikeda K, Kamiya K (2016) In vitro models of GJB2-Related hearing loss recapitulate Ca2+ transients via a gap junction characteristic of developing Cochlea. Stem Cell Rep 7(6):1023–1036

Fung M, Babik JM (2020) COVID-19 in immunocompromised hosts: what we know so far. Clin Infect Dis 72(2):340–350

Fuster-García C, García-García G, González-Romero E, Jaijo T, Sequedo MD, Ayuso C, Vázquez-Manrique RP, Millán JM, Aller E (2017) USH2A gene editing using the CRISPR system. Mol Ther Nucleic Acids 8:529–541

Galougahi MK, Ghorbani J, Bakhshayeshkaram M, Naeini AS, Haseli S (2020) Olfactory bulb magnetic resonance imaging in SARS-CoV-2-induced anosmia: the first report. Acad Radiol

Gálvez H, Abelló G, Giraldez F (2017) Signaling and transcription factors during inner ear development: the generation of hair cells and otic neurons. Front Cell Dev Biol 5:21

Ganapathy A, Pandey N, Srisailapathy CS, Jalvi R, Malhotra V, Venkatappa M, Chatterjee A, Sharma M, Santhanam R, Chadha S, Ramesh A (2014) Non-syndromic hearing impairment in India: high allelic heterogeneity among mutations in TMPRSS3, TMC1, USHIC, CDH23 and TMIE. PLoS One 9(1):e84773

Gao X, Huang SS, Yuan YY, Wang GJ, Xu JC, Ji YB, Han MY, Yu F, Kang DY, Lin X, Dai P (2015) Targeted gene capture and massively parallel sequencing identify TMC1 as the causative

gene in a six-generation Chinese family with autosomal dominant hearing loss. Am J Med Genet A 167(10):2357–2365
Gao L, Guan W, Wang M, Wang H, Yu J, Liu Q, Qiu B, Yu Y, Ping Y, Bian X, Shen L (2017) Direct generation of human neuronal cells from adult astrocytes by small molecules. Stem Cell Rep 8(3):538–547
Garg S, Chadha S, Malhotra S, Agarwal AK (2009) Deafness: burden, prevention and control in India. Natl Med J India 22(2):79–81
Ge X, Jackson RL, Liu J, Harper EA, Hoffer ME, Wassel RA, Dormer KJ, Kopke RD, Balough BJ (2007) Distribution of PLGA nanoparticles in chinchilla cochleae. Otolaryngology—Head and Neck. Surgery 137(4):619–623
Géléoc GS, Holt JR (2014) Sound strategies for hearing restoration. Science 344(6184):1241062
Geng R, Furness DN, Muraleedharan CK, Zhang J, Dabdoub A, Lin V, Xu S (2018) The microRNA-183/96/182 cluster is essential for stereociliary bundle formation and function of cochlear sensory hair cells. Sci Rep 8(1):1–3
Giacomelli A, Pezzati L, Conti F, Bernacchia D, Siano M, Oreni L, Rusconi S, Gervasoni C, Ridolfo AL, Rizzardini G, Antinori S (2020) Self-reported olfactory and taste disorders in patients with severe acute respiratory coronavirus 2 infection: a cross-sectional study. Clin Infect Dis 71(15):889–890
Goman AM, Lin FR (2016) Prevalence of hearing loss by severity in the United States. Am J Public Health 106(10):1820–1822
Goycoolea MV, Lundman L (1997) Round window membrane. Structure function and permeability: a review. Microsc Res Tech 36(3):201–211
Green GE, Scott DA, McDonald JM, Teagle HF, Tomblin BJ, Spencer LJ, Woodworth GG, Knutson JF, Gantz BJ, Sheffield VC, Smith RJ (2002) Performance of cochlear implant recipients with GJB2-related deafness. Am J Med Genet 109(3):167–170
Grifoni A, Sidney J, Zhang Y, Scheuermann RH, Peters B, Sette A (2020) A sequence homology and bioinformatic approach can predict candidate targets for immune responses to SARS-CoV-2. Cell Host Microbe 27(4):671–680.e2
Gruenert DC, Basbaum CB, Widdicombe JH (1990) Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. In Vitro Cell Dev Biol 26(4):411–418
Guo H, Pu X, Chen J, Meng Y, Yeh MH, Liu G, Tang Q, Chen B, Liu D, Qi S, Wu C (2018) A highly sensitive, self-powered triboelectric auditory sensor for social robotics and hearing aids. Sci Robot 3(20)
Haehner A, Draf J, Draeger S, de With K, Hummel T (2020) Predictive value of sudden olfactory
Hannum ME, Ramirez VA, Lipson SJ, Herriman RD, Toskala AK, Lin C, Joseph PV, Reed DR (2020) Objective sensory testing methods reveal a higher prevalence of olfactory loss in COVID-19–positive patients compared to subjective methods: a systematic review and meta-analysis. MedRxiv 6
Hao Y, Chen D, Zhang Z, Zhou P, Cao Y, Wei Z, Xu X, Chen B, Zou W, Lv M, Ji D (2018) Successful preimplantation genetic diagnosis by targeted next-generation sequencing on an ion torrent personal genome machine platform. Oncol Lett 15(4):4296–4302
Hartwell RD, England SJ, Monk NA, Van Hateren NJ, Baxendale S, Marzo M, Lewis KE, Whitfield TT (2019) Anteroposterior patterning of the zebrafish ear through Fgf-and Hh-dependent regulation of hmx3a expression. PLoS Genet 15(4):e1008051
Hassan MA, Shah AA, Szmida E, Smigiel R, Sasiadek MM, Pfister M, Blin N, Bress A (2015) A TMC1 (transmembrane channel-like 1) mutation (p. S320R) in a Polish family with hearing impairment. J Appl Genet 56(3):311–316
Hoijman E, Fargas L, Blader P, Alsina B (2017) Pioneer neurog1 expressing cells ingress into the otic epithelium and instruct neuronal specification. elife 6:e25543
Hopkins C, Surda P, Kumar N (2020) Presentation of new onset anosmia during the COVID-19 pandemic. Rhinology 11:10
Hornuss D, Lange B, Schröter N, Rieg S, Kern WV, Wagner D (2020) Anosmia in COVID-19 patients. Clin Microbiol Infect

Hota A (2019) Factors affecting audiological performance and speech intelligibility in prelingually deaf children after cochlear implantation: a study. Int J Otorhinolaryngol Head Neck Surg 5(4): 1035.21; 303(15):1498–1506

Hou Y, Zhao J, Martin W, Kallianpur A, Chung MK, Jehi L, Sharifi N, Erzurum S, Eng C, Cheng F (2020) New insights into genetic susceptibility of COVID-19: an ACE2 and TMPRSS2 polymorphism analysis. BMC Med 18(1):1–8

Hu Z, Luo X, Zhang L, Lu F, Dong F, Monsell E, Jiang H (2012) Generation of human inner ear prosensory-like cells via epithelial-to-mesenchymal transition. Regen Med 7(5):663–673

Huang S, Wiszniewski L, Constant S (2011) The use of in vitro 3D cell models in drug development for respiratory diseases, vol 8, Chapter

Huang S, Boda B, Vernaz J, Ferreira E, Wiszniewski L, Constant S (2017) Establishment and characterization of an in vitro human small airway model (SmallAir). Eur J Pharm Biopharm 118:68–72

Huber M, Roesch S, Pletzer B, Lukaschyk J, Lesinski-Schiedat A, Illg A (2020) Cognition in older adults with severe to profound sensorineural hearing loss compared to peers with normal hearing for age. Int J Audiol 59(4):254–262

Illum L (2002) Nasal drug delivery: new developments and strategies. Drug Discov Today 7(23): 1184–1189

Illum L (2003) Nasal drug delivery—possibilities, problems and solutions. J Control Release 87(1-3):187–198

Iravani B, Arshamian A, Ravia A, Mishor E, Snitz K, Shushan S, Roth Y, Perl O, Honigstein D, Weissgross R, Karagach S (2020) Relationship between odor intensity estimates and COVID-19 prevalence prediction in a Swedish population. Chem Senses

Jayakody DM, Almeida OP, Speelman CP, Bennett RJ, Moyle TC, Yiannos JM, Friedland PL (2018) Association between speech and high-frequency hearing loss and depression, anxiety and stress in older adults. Maturitas 110:86–91

Johnson PH, Quay SC (2005) Advances in nasal drug delivery through tight junction technology. Expert Opin Drug Deliv 2(2):281–298

Johnson PH, Frank D, Costantino HR (2008) Discovery of tight junction modulators: significance for drug development and delivery. Drug Discov Today 13(5–6):261–267

Jolly C, Garnham C, Mirzadeh H, Truy E, Martini A, Kiefer J, Braun S (2010) Electrode features for hearing preservation and drug delivery strategies. In: Cochlear implants and hearing preservation, vol 67. Karger Publishers, pp 28–42

Kandimalla KK, Donovan MD (2005) Carrier mediated transport of chlorpheniramine and chlorcyclizine across bovine olfactory mucosa: implications on nose-to-brain transport. J Pharm Sci 94(3):613–624

Karamert R, Bayazit YA, Altinyay S, Yılmaz A, Menevse A, Gokdogan O, Gokdogan C, Ant A (2011) Association of GJB2 gene mutation with cochlear implant performance in genetic non-syndromic hearing loss. Int J Pediatr Otorhinolaryngol 75(12):1572–1575

Kaye R, Chang CD, Kazahaya K, Brereton J, Denneny JC III (2020) COVID-19 anosmia reporting tool: initial findings. Otolaryngol Head Neck Surg 163(1):132–134. https://doi.org/10.1177/0194599820922992

Kim D-D (2008) In vitro cellular models for nasal drug absorption studies. Drug absorption studies. Springer, pp 216–234

Kitano T, Miyagawa M, Nishio SY, Moteki H, Oda K, Ohyama K, Miyazaki H, Hidaka H, Nakamura KI, Murata T, Matsuoka R (2017) POU4F3 mutation screening in Japanese hearing loss patients: massively parallel DNA sequencing-based analysis identified novel variants associated with autosomal dominant hearing loss. PLoS One 12(5):e0177636

Koehler KR, Hashino E (2014) 3D mouse embryonic stem cell culture for generating inner ear organoids. Nat Protoc 9(6):1229

Koehler KR, Mikosz AM, Molosh AI, Patel D, Hashino E (2013) Generation of inner ear sensory epithelia from pluripotent stem cells in 3D culture. Nature 500(7461):217–221

Koehler KR, Nie J, Longworth-Mills E, Liu XP, Lee J, Holt JR, Hashino E (2017) Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells. Nat Biotechnol 35(6):583

Kremer H (2019) Hereditary hearing loss; about the known and the unknown. Hear Res 376:58–68

Kuchay RA, Mir YR, Zeng X, Hassan A, Namba K, Tekin M (2020) Novel OTOF pathogenic variant segregating with non-syndromic hearing loss in a consanguineous family from tribal Rajouri in Jammu and Kashmir. Int J Pediatr Otorhinolaryngol 130:109831

Kumar RN, Kameswaran M (2019) Practical and ethical issues for delivery of advanced ENT services in the Indian subcontinent: efforts to 'Bridge the Gap'. Curr Opin Otolaryngol Head Neck Surg 27(3):212–218

Ladel S, Schlossbauer P, Flamm J, Luksch H, Mizaikoff B, Schindowski K (2019) Improved in vitro model for intranasal mucosal drug delivery: primary olfactory and respiratory epithelial cells compared with the permanent nasal cell line RPMI 2650. Pharmaceutics 11(8)

Ladher RK, Wright TJ, Moon AM, Mansour SL, Schoenwolf GC (2005) FGF8 initiates inner ear induction in chick and mouse. Genes Dev 19(5):603–613

Ladher RK, O'Neill P, Begbie J (2010) From shared lineage to distinct functions: the development of the inner ear and epibranchial placodes. Development 137(11):1777–1785

Lahlou H, Lopez-Juarez A, Fontbonne A, Nivet E, Zine A (2018) Modeling human early otic sensory cell development with induced pluripotent stem cells. PLoS One 13(6):e0198954

Lalwani AK, McGuire JF (2005) Pharmacologic treatment of the cochlea and labyrinth. Otolaryngology-Head and Neck Surgery, 4th edn. Elsevier Mosby, Philadelphia

Landegger LD, Pan B, Askew C, Wassmer SJ, Gluck SD, Galvin A, Taylor R, Forge A, Stankovic KM, Holt JR, Vandenberghe LH (2017) A synthetic AAV vector enables safe and efficient gene transfer to the mammalian inner ear. Nat Biotechnol 35(3):280–284

Lawrence BJ, Jayakody DM, Henshaw H, Ferguson MA, Eikelboom RH, Loftus AM, Friedland PL (2018) Auditory and cognitive training for cognition in adults with hearing loss: a systematic review and meta-analysis. Trends Hear 22:2331216518792096

Lawrence BJ, Jayakody DM, Bennett RJ, Eikelboom RH, Gasson N, Friedland PL (2020) Hearing loss and depression in older adults: a systematic review and meta-analysis. The Gerontologist 60(3):e137–e154

Lechien JR, Chiesa-Estomba CM, De Siati DR, Horoi M, Le Bon SD, Rodriguez A, Dequanter D, Blecic S, El Afia F, Distinguin L, Chekkoury-Idrissi Y (2020) Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. Eur Arch Otorhinolaryngol 6:1–1

Lee MK, Yoo JW, Lin H et al (2005) Air-liquid interface culture of serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. Drug Deliv 12(5):305–311

Lee HK, Lee SH, Lee KY, Lim EJ, Choi SY, Park RK, Kim UK (2009) Novel POU3F4 mutations and clinical features of DFN3 patients with cochlear implants. Clin Genet 75(6):572–575

Lee HS, Kim WJ, Gong JS, Park KH (2018) Clinical safety and efficacy of autologous bone marrow-derived mesenchymal stem cell transplantation in sensorineural hearing loss patients. J Audiol Otol 22(2):105

Lenz DR, Gunewardene N, Abdul-Aziz DE, Wang Q, Gibson TM, Edge AS (2019) Applications of Lgr5-positive cochlear progenitors (LCPs) to the study of hair cell differentiation. Front Cell Dev Biol 7:14

Leonard AK, Sileno AP, Brandt GC, Foerder CA, Quay SC, Costantino HR (2007) In vitro formulation optimization of intranasal galantamine leading to enhanced bioavailability and reduced emetic response in vivo. Int J Pharm 335(1–2):138–146

Lewis MA, Nolan LS, Cadge BA, Matthews LJ, Schulte BA, Dubno JR, Steel KP, Dawson SJ (2018) Whole exome sequencing in adult-onset hearing loss reveals a high load of predicted pathogenic variants in known deafness-associated genes and identifies new candidate genes. BMC Med Genet 11(1):77

Li G, De Clercq E (2020) Therapeutic options for the 2019 novel coronavirus (2019-nCoV). Nat Rev Drug Discov 19(3):149–150

Li H, Liu H, Heller S (2003) Pluripotent stem cells from the adult mouse inner ear. Nat Med 9(10): 1293–1299

Li H, Feng G, Wang H, Feng Y (2015) Intratympanic steroid therapy as a salvage treatment for sudden sensorineural hearing loss after failure of conventional therapy: a meta-analysis of randomized, controlled trials. Clin Ther 37(1):178–187

Liang H, Acharya G (2020) Novel corona virus disease (COVID-19) in pregnancy: what clinical recommendations to follow? Acta Obstet Gynecol Scand 99(4):439–442

Lin H, Yoo JW, Roh HJ et al (2005) Transport of anti-allergic drugs across the passage cultured human nasal epithelial cell monolayer. Eur J Pharm Sci 26(2):203–210

Lin FR, Niparko JK, Ferrucci L (2011) Hearing loss prevalence in the United States. Arch Intern Med 171(20):1851–1853

Lipworth BJ, Jackson CM (2000) Safety of inhaled and intranasal corticosteroids: lessons for the new millennium. Drug Saf 23(1):11–33

Liu C, Luo N, Tung CY, Perrin BJ, Zhao B (2018) GRXCR2 regulates taperin localization critical for stereocilia morphology and hearing. Cell Rep 25(5):1268–1280

Liu Y, Gayle AA, Wilder-Smith A, Rocklöv J (2020) The reproductive number of COVID-19 is higher compared to SARS coronavirus. J Travel Med 27(2):taaa021

Longworth-Mills E, Koehler KR, Hashino E (2015) Generating inner ear organoids from mouse embryonic stem cells. In: Embryonic stem cell protocols. Humana Press, New York, pp 391–406

Lopez-Juarez A, Lahlou H, Ripoll C, Cazals Y, Brezun JM, Wang Q, Edge A, Zine A (2019) Engraftment of human stem cell-derived Otic progenitors in the damaged cochlea. Mol Ther 27(6):1101–1113

Luo S, Hu D, Wang M, Zipfel PF, Hu Y (2020) Complement in hemolysis-and thrombosis-related diseases. Front Immunol 11

Mäder K, Lehner E, Liebau A, Plontke SK (2018) Controlled drug release to the inner ear: concepts, materials, mechanisms, and performance. Hear Res 368:49–66

Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, Chang J, Hong C, Zhou Y, Wang D, Miao X (2020) Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. JAMA Neurol 77(6):683–690

Maoiléidigh DÓ, Ricci AJ (2019) A bundle of mechanisms: inner-ear hair-cell mechanotransduction. Trends Neurosci 42(3):221–236

Matsuoka K, Wada K, Miyasaka Y, Yasuda SP, Seki Y, Nishito Y, Yonekawa H, Taya C, Shitara H, Kikkawa Y (2019) OHC-TRECK: a novel system using a mouse model for investigation of the molecular mechanisms associated with outer hair cell death in the inner ear. Sci Rep 9(1):1–7

Matsushiro N, Doi K, Fuse Y, Nagai K, Yamamoto K, Iwaki T, Kawashima T, Sawada A, Hibino H, Kubo T (2002) Successful cochlear implantation in prelingual profound deafness resulting from the common 233delC mutation of the GJB2 gene in the Japanese. Laryngoscope 112(2):255–261

Mattinger C, Nyugen T, Schafer D, Hormann K (2002) Evaluation of serum-free culture conditions for primary human nasal epithelial cells. Int J Hyg Environ Health 205(3):235–238

McElvaney OJ, McEvoy NL, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, Ní Choileáin O, Clarke J, O'Connor E, Hogan G, Ryan D (2020) Characterization of the inflammatory response to severe COVID-19 illness. Am J Respir Crit Care Med 202(6):812–821

McGovern MM, Randle MR, Cuppini CL, Graves KA, Cox BC (2019) Multiple supporting cell subtypes are capable of spontaneous hair cell regeneration in the neonatal mouse cochlea. Development 146(4)

McLean WJ, Yin X, Lu L, Lenz DR, McLean D, Langer R, Karp JM, Edge AS (2017) Clonal expansion of Lgr5-positive cells from mammalian cochlea and high-purity generation of sensory hair cells. Cell Rep 18(8):1917–1929

Meas SJ, Zhang CL, Dabdoub A (2018) Reprogramming glia into neurons in the peripheral auditory system as a solution for sensorineural hearing loss: lessons from the central nervous system. Front Mol Neurosci 11:77

Mehregan H, Mohseni M, Jalalvand K, Arzhangi S, Nikzat N, Banihashemi S, Kahrizi K, Najmabadi H (2019) Novel mutations in MYTH4-FERM domains of myosin 15 are associated with autosomal recessive nonsyndromic hearing loss. Int J Pediatr Otorhinolaryngol 117:115–126

Meinhardt J, Radke J, Dittmayer C, Mothes R, Franz J, Laue M, Schneider J, Bruenink S, Hassan O, Stenzel W, Windgassen M (2020) Olfactory transmucosal SARS-CoV-2 invasion as port of Central Nervous System entry in COVID-19 patients. bioRxiv

Menni C, Valdes AM, Freidin MB, Sudre CH, Nguyen LH, Drew DA, Ganesh S, Varsavsky T, Cardoso MJ, Moustafa JS, Visconti A (2020) Real-time tracking of self-reported symptoms to predict potential COVID-19. Nat Med 11:1–4

Merkus FW, Verhoef JC, Schipper NG, Marttin E (1998) Nasal mucociliary clearance as a factor in nasal drug delivery. Adv Drug Deliv Rev 29(1–2):13–38

Miyagawa M, Nishio SY, Ikeda T, Fukushima K, Usami SI (2013) Massively parallel DNA sequencing successfully identifies new causative mutations in deafness genes in patients with cochlear implantation and EAS. PLoS One 8(10):e75793

Miyagawa M, Nishio SY, Kumakawa K, Usami SI (2015) Massively parallel DNA sequencing successfully identified seven families with deafness-associated MYO6 mutations: the mutational spectrum and clinical characteristics. Ann Otol Rhinol Laryngol 124(1_Suppl):148S–157S

Miyagawa M, Nishio SY, Usami SI (2016) A comprehensive study on the etiology of patients receiving cochlear implantation with special emphasis on genetic epidemiology. Otol Neurotol 37(2):e126

Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, Xiong Y, Cheng Z, Gao S, Liang K, Luo M (2020) Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. Clin Infect Dis

Moein ST, Hashemian SM, Mansourafshar B, Khorram-Tousi A, Tabarsi P, Doty RL (2020) Smell dysfunction: a biomarker for COVID-19. Int Forum Allergy Rhinol

Mori K, Moteki H, Kobayashi Y, Azaiez H, Booth KT, Nishio SY, Sato H, Smith RJ, Usami SI (2015) Mutations in LOXHD1 gene cause various types and severities of hearing loss. Ann Otol Rhinol Laryngol 124(1_Suppl):135S–141S

Morrison EE, Costanzo RM (1990) Morphology of the human olfactory epithelium. J Comp Neurol 297(1):1–13

Morton CC, Nance WE (2006) Newborn hearing screening—a silent revolution. N Engl J Med 354(20):2151–2164

Müller U, Barr-Gillespie PG (2015) New treatment options for hearing loss. Nat Rev Drug Discov 14(5):346–365

Munnamalai V, Fekete DM (2017) Building the human inner ear in an organoid. Nat Biotechnol 35(6):518

Munster VJ, Feldmann F, Williamson BN, van Doremalen N, Pérez-Pérez L, Schulz J, Meade-White K, Okumura A, Callison J, Brumbaugh B, Avanzato VA (2020) Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. Nature 585(7824):268–272

Mygind N, Dahl R (1998) Anatomy, physiology and function of the nasal cavities in health and disease. Adv Drug Deliv Rev 29(1–2):3–12

Nakanishi H, Kurima K, Kawashima Y, Griffith AJ (2014) Mutations of TMC1 cause deafness by disrupting mechanoelectrical transduction. Auris Nasus Larynx 41(5):399–408

Negri EM, Piloto B, Morinaga LK, Jardim CV, Lamy SA, Ferreira MA, D'Amico EA, Deheinzelin D (2020) Heparin therapy improving hypoxia in COVID-19 patients-a case series. medRxiv

Niclasen J, Obel C, Homøe P, Kørvel-Hanquist A, Dammeyer J (2016) Associations between otitis media and child behavioural and learning difficulties: results from a Danish cohort. Int J Pediatr Otorhinolaryngol 84:12–20

Niparko JK, Tobey EA, Thal DJ, Eisenberg LS, Wang NY, Quittner AL, Fink NE (2010) CDaCI Investigative Team. Spoken language development in children following cochlear implantation. JAMA 303(15):1498–1506

Nishio SY, Usami SI (2015) Deafness gene variations in a 1120 nonsyndromic hearing loss cohort: molecular epidemiology and deafness mutation spectrum of patients in Japan. Ann Otol Rhinol Laryngol 124(1_Suppl):49S–60S

Nishiyama N, Kawano A, Kawaguchi S, Shirai K, Suzuki M (2013) Cochlear implantation in a patient with Epstein syndrome. Auris Nasus Larynx 40(4):409–412

Nist-Lund CA, Pan B, Patterson A, Asai Y, Chen T, Zhou W, Zhu H, Romero S, Resnik J, Polley DB, Géléoc GS (2019) Improved TMC1 gene therapy restores hearing and balance in mice with genetic inner ear disorders. Nat Commun 10(1):1–4

Ogier JM, Burt RA, Drury HR, Lim R, Nayagam BA (2019) Organotypic culture of neonatal murine inner ear explants. Front Cell Neurosci 13:170

Oguntuyo KY, Stevens CS, Siddiquey MN, Schilke RM, Woolard MD, Zhang H, Acklin JA, Ikegame S, Huang CT, Lim JK, Cross RW (2020) In plain sight: the role of alpha-1-antitrypsin in COVID-19 pathogenesis and therapeutics. bioRxiv

Ohnishi H, Skerleva D, Kitajiri SI, Sakamoto T, Yamamoto N, Ito J, Nakagawa T (2015) Limited hair cell induction from human induced pluripotent stem cells using a simple stepwise method. Neurosci Lett 599:49–54

Olusanya BO, Neumann KJ, Saunders JE (2014) The global burden of disabling hearing impairment: a call to action. Bull World Health Organ 92:367–373

Ono K, Kita T, Sato S, O'Neill P, Mak SS, Paschaki M, Ito M, Gotoh N, Kawakami K, Sasai Y, Ladher RK (2014) FGFR1-Frs2/3 signalling maintains sensory progenitors during inner ear hair cell formation. PLoS Genet 10(1):e1004118

Oonk AM, Leijendeckers JM, Lammers EM, Weegerink NJ, Oostrik J, Beynon AJ, Huygen PL, Kunst HP, Kremer H, Snik AF, Pennings RJ (2013) Progressive hereditary hearing impairment caused by a MYO6 mutation resembles presbyacusis. Hear Res 299:88–98

Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S (2010) Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell 141(4):704–716

Pan B, Akyuz N, Liu XP, Asai Y, Nist-Lund C, Kurima K, Derfler BH, György B, Limapichat W, Walujkar S, Wimalasena LN (2018) TMC1 forms the pore of mechanosensory transduction channels in vertebrate inner ear hair cells. Neuron 99(4):736–753

Pandey N, Rashid T, Jalvi R, Sharma M, Rangasayee R, Andrabi KI, Anand A (2017) Mutations in OTOF, CLDN14 & SLC26A4 genes as major causes of hearing impairment in Dhadkai village, Jammu & Kashmir, India. Indian J Med Res 146(4):489

Pararas EE, Borkholder DA, Borenstein JT (2012) Microsystems technologies for drug delivery to the inner ear. Adv Drug Deliv Rev 64(14):1650–1660

Park H, Hong SN, Kim HS, Han JJ, Chung J, Seo MW, Oh SH, Chang SO, Lee JH (2015) Determinants of conductive hearing loss in tympanic membrane perforation. Clin Exp Otorhinolaryngol 8(2):92

Parma V, Ohla K, Veldhuizen MG, Niv MY, Kelly CE, Bakke AJ, Cooper KW, Bouysset C, Pirastu N, Dibattista M, Kaur R (2020) More than smell—COVID-19 is associated with severe impairment of smell, taste, and chemesthesis. Chem Senses 45(7):609–622

Passarelli PC, Lopez MA, Bonaviri GM, Garcia-Godoy F, D'Addona A (2020) Taste and smell as chemosensory dysfunctions in COVID-19 infection. Am J Dent 33(3):135–137

Pauw RJ, van Drunen FW, Collin RW, Huygen PL, Kremer H, Cremers CW (2008) Audiometric characteristics of a Dutch family linked to DFNA15 with a novel mutation (p. L289F) in POU4F3. Arch Otolaryngol Head Neck Surg 134(3):294–300

Pecci A, Verver EJ, Schlegel N, Canzi P, Boccio CM, Platokouki H, Krause E, Benazzo M, Topsakal V, Greinacher A (2014) Cochlear implantation is safe and effective in patients with MYH9-related disease. Orphanet J Rare Dis 9(1):100

Perny M, Ting CC, Kleinlogel S, Senn P, Roccio M (2017) Generation of otic sensory neurons from mouse embryonic stem cells in 3D culture. Front Cell Neurosci 11:409

Peterson B, Weyers M, Steenekamp JH, Steyn JD, Gouws C, Hamman JH (2019) Drug bioavailability enhancing agents of natural origin (bioenhancers) that modulate drug membrane permeation and pre-systemic metabolism. Pharmaceutics 11(1)

Pezzini A, Padovani A (2020) Lifting the mask on neurological manifestations of COVID-19. Nat Rev Neurol 16(11):636–644

Pires A, Fortuna A, Alves G, Falcao A (2009) Intranasal drug delivery: how, why and what for? J Pharm Pharm Sci 12(3):288–311

Plontke SK (2017) Diagnostics and therapy of sudden hearing loss. GMS Curr Topics Otorhinolaryngol Head Neck Surg 16

Plontke SK, Hartsock JJ, Gill RM, Salt AN (2016) Intracochlear drug injections through the round window membrane: measures to improve drug retention. Audiol Neurotol 21(2):72–79

Pollak A, Lechowicz U, Kędra A, Stawiński P, Rydzanicz M, Furmanek M, Brzozowska M, Mrówka M, Skarżyński H, Skarżyński PH, Ołdak M (2016) Novel and de novo mutations extend association of POU3F4 with distinct clinical and radiological phenotype of hearing loss. PLoS One 11(12):e0166618

Printza A, Constantinidis J (2020) The role of self-reported smell and taste disorders in suspected COVID-19. Eur Arch Otorhinolaryngol 23:1–6

Pritz CO, Dudás J, Rask-Andersen H, Schrott-Fischer A, Glueckert R (2013) Nanomedicine strategies for drug delivery to the ear. Nanomedicine 8(7):1155–1172

Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS (2020) Dysregulation of immune response in patients with COVID-19 in Wuhan, China. Clin Infect Dis

Raft S, Groves AK (2015) Segregating neural and mechanosensory fates in the developing ear: patterning, signaling, and transcriptional control. Cell Tissue Res 359(1):315–332

Ritter KE, Martin DM (2019) Neural crest contributions to the ear: implications for congenital hearing disorders. Hear Res 376:22–32

Rivolta MN, Li H, Heller S (2006) Generation of inner ear cell types from embryonic stem cells. In: Embryonic stem cell protocols. Humana Press, pp 71–92

Roccio M, Edge AS (2019) Inner ear organoids: new tools to understand neurosensory cell development, degeneration and regeneration. Development 146(17):dev177188

Roccio M, Perny M, Ealy M, Widmer HR, Heller S, Senn P (2018) Molecular characterization and prospective isolation of human fetal cochlear hair cell progenitors. Nat Commun 9(1):1–4

Rodriguez S, Cao L, Rickenbacher GT, Benz EG, Magdamo C, Gomez LA, Holbrook E, Albers AD, Gallagher R, Westover MB, Evans KE (2020) Innate immune signaling in the olfactory epithelium reduces odorant receptor levels: modeling transient smell loss in COVID-19 patients. medRxiv

Rodriguez-Morales AJ, Cardona-Ospina JA, Gutiérrez-Ocampo E, Villamizar-Peña R, Holguin-Rivera Y, Escalera-Antezana JP, Alvarado-Arnez LE, Bonilla-Aldana DK, Franco-Paredes C, Henao-Martinez AF, Paniz-Mondolfi A (2020) Clinical, laboratory and imaging features of COVID-19: a systematic review and meta-analysis. Travel Med Infect Dis 13:101623

Roemer A, Köhl U, Majdani O, Klöß S, Falk C, Haumann S, Lenarz T, Kral A, Warnecke A (2016) Biohybrid cochlear implants in human neurosensory restoration. Stem Cell Res Ther 7(1):1–4

Roland JT Jr, Gantz BJ, Waltzman SB, Parkinson AJ (2016) Multicenter Clinical Trial Group. United States multicenter clinical trial of the cochlear nucleus hybrid implant system. Laryngoscope 126(1):175–181

Ronaghi M, Nasr M, Ealy M, Durruthy-Durruthy R, Waldhaus J, Diaz GH, Joubert LM, Oshima K, Heller S (2014) Inner ear hair cell-like cells from human embryonic stem cells. Stem Cells Dev 23(11):1275–1284

Rouillon I, Marcolla A, Roux I, Marlin S, Feldmann D, Couderc R, Jonard L, Petit C, Denoyelle F, Garabedian EN, Loundon N (2006) Results of cochlear implantation in two children with mutations in the OTOF gene. Int J Pediatr Otorhinolaryngol 70(4):689–696

Roy P, Perrin BJ (2018) The stable actin core of mechanosensory stereocilia features continuous turnover of actin cross-linkers. Mol Biol Cell 29(15):1856–1865

Rudman JR, Mei C, Bressler SE, Blanton SH, Liu XZ (2018) Precision medicine in hearing loss. J Genet Genomics 45(2):99–109

Sakuma N, Moteki H, Azaiez H, Booth KT, Takahashi M, Arai Y, Shearer AE, Sloan CM, Nishio SY, Kolbe DL, Iwasaki S (2015) Novel PTPRQ mutations identified in three congenital hearing loss patients with various types of hearing loss. Ann Otol Rhinol Laryngol 124(1_Suppl):184S–192S

Sanggaard KM, Kjaer KW, Eiberg H, Nürnberg G, Nürnberg P, Hoffman K, Jensen H, Sørum C, Rendtorff ND, Tranebjærg L (2008) A novel nonsense mutation in MYO6 is associated with progressive nonsyndromic hearing loss in a Danish DFNA22 family. Am J Med Genet A 146(8):1017–1025

Saussez S, Lechien JR, Hopkins C (2020) Anosmia: an evolution of our understanding of its importance in COVID-19 and what questions remain to be answered. Eur Arch Otorhinolaryngol 9:1–5

Schaefer SA, Higashi AY, Loomis B, Schrepfer T, Wan G, Corfas G, Dressler GR, Duncan RK (2018) From otic induction to hair cell production: Pax2EGFP cell line illuminates key stages of development in mouse inner ear organoid model. Stem Cells Dev 27(4):237–251

Schilder AG, Su MP, Blackshaw H, Lustig L, Staecker H, Lenarz T, Safieddine S, Gomes-Santos CS, Holme R, Warnecke A (2019) Hearing protection, restoration, and regeneration: an overview of emerging therapeutics for inner ear and central hearing disorders. Otol Neurotol 40(5):559–570

Schipper NG, Verhoef JC, Merkus FW (1991) The nasal mucociliary clearance: relevance to nasal drug delivery. Pharm Res 8(7):807–814

Schmaier AH (2019) Serpin targets in hemostasis/kinin formation. Blood 134(19):1566–1568

Sedaghat A, Oloomi SA, Malayer MA, Rezaei N, Mosavi A (2020) Coronavirus (COVID-19) outbreak prediction using epidemiological models of Richards Gompertz Logistic Ratkowsky and SIRD. medRxiv

Selivanova O, Maurer J, Ecke U, Mann WJ (2003) The effects of Streptolysin-O and sodium hyaluronate on the permeability of the round window membrane in guinea pigs—an electrophysiologic study. Laryngo-Rhino-Otologie 82(4):235–239

Senn P, Mina A, Volkenstein S, Kranebitter V, Oshima K, Heller S (2020) Progenitor cells from the adult human inner ear. Anat Rec 303(3):461–470

Shapira G, Shomron N, Gurwitz D (2020) Ethnic differences in alpha-1 antitrypsin deficiency allele frequencies may partially explain national differences in COVID-19 fatality rates. FASEB J 34(11):14160–14165

Sharma M, Panda NK (2020) Proteomic profiling of protease-primed virus-permissive Caco-2 cells display abortive-interferon pathway and deregulated thromboinflammatory SERPINS. Preprints

Sharma M, Lei H, Pennock S, Kazlauskas A (2013) Epithelial cells promote fibroblast-mediated contraction of collagen gels by secreting bFGF. Investig Ophthalmol Vis Sci 54(15):6258

Sharma M, Kumar R, Ram J, Luthra-Guptasarma M (2017) Establishment and characterization of a novel Serine Protease Induced Reprograming (SPIR) method with applications in ocular tissue regeneration. Invest Ophthalmol Vis Sci 58(8):1426

Sharma M, Kumar R, Sharma S, Thomas B, Kapatia G, Singh G, Bal A, Ram J, Bhasin M, Guptasarma P, Luthra-Guptasarma M (2019) Sustained exposure to trypsin causes cells to transition into a state of reversible stemness that is amenable to transdifferentiation. bioRxiv 1:679928

Sharma M, Kaushal K, Rawat SS, Muraleedharan M, Chhabra S, Verma N, Mittal A, Bahl A, Khullar M, Ramavat A, Panda NK (2021) The cellular stress response interactome and extracellular matrix cross-talk during fibrosis: a stressed extra-matrix affair. https://doi.org/10.5772/intechopen.95066

Shearer AE, DeLuca AP, Hildebrand MS, Taylor KR, Gurrola J, Scherer S, Scheetz TE, Smith RJ (2010) Comprehensive genetic testing for hereditary hearing loss using massively parallel sequencing. Proc Natl Acad Sci 107(49):21104–21109

Shearer AE, Kolbe DL, Azaiez H, Sloan CM, Frees KL, Weaver AE, Clark ET, Nishimura CJ, Black-Ziegelbein EA, Smith RJ (2014) Copy number variants are a common cause of non-syndromic hearing loss. Genome Med 6(5):37

Shearer AE, Hildebrand MS, Smith RJ (2017) Hereditary hearing loss and deafness overview. In: GeneReviews® [Internet]. University of Washington, Seattle

Shen B, Yi X, Sun Y, Bi X, Du J, Zhang C, Quan S, Zhang F, Sun R, Qian L, Ge W (2020) Proteomic and metabolomic characterization of COVID-19 patient sera. Cell 182(1):59–72

Shi F, Kempfle JS, Edge AS (2012) Wnt-responsive Lgr5-expressing stem cells are hair cell progenitors in the cochlea. J Neurosci 32(28):9639–9648

Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, Liu R, He X, Shuai L, Sun Z, Zhao Y (2020) Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS–coronavirus 2. Science 368(6494):1016–1020

Shoer S, Karady T, Keshet A, Shilo S, Rossman H, Gavrieli A, Meir T, Lavon A, Kolobkov D, Kalka I, Godneva A (2020) Who should we test for COVID-19? A triage model built from national symptom surveys. medRxiv

Shweta FN, Murugadoss K, Awasthi S, Venkatakrishnan AJ, Puranik A, Kang M, Pickering BW, O'Horo JC, Bauer PR, Razonable RR, Vergidis P (2020) Augmented curation of unstructured clinical notes from a massive EHR system reveals specific phenotypic signature of impending COVID-19 diagnosis. arXiv preprint arXiv:2004.09338

Sia SF, Yan LM, Chin AW, Fung K, Choy KT, Wong AY, Kaewpreedee P, Perera RA, Poon LL, Nicholls JM, Peiris M (2020) Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. Nature 583(7818):834–838

Silverstein H, Thompson J, Rosenberg SI, Brown N, Light J (2004) Silverstein MicroWick. Otolaryngol Clin N Am 37(5):1019

Sinnathuray AR, Toner JG, Clarke-Lyttle J, Geddis A, Patterson CC, Hughes AE (2004) Connexin 26 (GJB2) gene-related deafness and speech intelligibility after cochlear implantation. Otol Neurotol 25(6):935–942

Sloan-Heggen CM, Bierer AO, Shearer AE, Kolbe DL, Nishimura CJ, Frees KL, Ephraim SS, Shibata SB, Booth KT, Campbell CA, Ranum PT (2016) Comprehensive genetic testing in the clinical evaluation of 1119 patients with hearing loss. Hum Genet 135(4):441–450

Spinato G, Fabbris C, Polesel J, Cazzador D, Borsetto D, Hopkins C, Boscolo-Rizzo P (2020) Alterations in smell or taste in mildly symptomatic outpatients with SARS-CoV-2 infection. JAMA 323(20):2089–2090

Stankovic KM, Hennessey AM, Herrmann B, Mankarious LA (2010) Cochlear implantation in children with congenital X-linked deafness due to novel mutations in POU3F4 gene. Ann Otol Rhinol Laryngol 119(12):815–822

Stevenson J, McCann D, Watkin P, Worsfold S, Kennedy C (2010) Hearing Outcomes Study Team. The relationship between language development and behaviour problems in children with hearing loss. J Child Psychol Psychiatry 51(1):77–83

Suckfuell M, Lisowska G, Domka W, Kabacinska A, Morawski K, Bodlaj R, Klimak P, Kostrica R, Meyer T (2014) Efficacy and safety of AM-111 in the treatment of acute sensorineural hearing loss: a double-blind, randomized, placebo-controlled phase II study. Otol Neurotol 35(8):1317–1326

Sun Y, Li L, Xie H et al (2020) Primary studies on construction and evaluation of ion-sensitive in situ gel loaded with paeonol-solid lipid nanoparticles for intranasal drug delivery. Int J Nanomedicine 15:3137–3160

Sungnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, Talavera-López C, Maatz H, Reichart D, Sampaziotis F, Worlock KB (2020) SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. Nat Med 26(5):681–687

Swan EE, Mescher MJ, Sewell WF, Tao SL, Borenstein JT (2008) Inner ear drug delivery for auditory applications. Adv Drug Deliv Rev 60(15):1583–1599

Tadenev AL, Akturk A, Devanney N, Mathur PD, Clark AM, Yang J, Tarchini B (2019) GPSM2-GNAI specifies the tallest stereocilia and defines hair bundle row identity. Curr Biol 29(6): 921–934

Takeda H, Miwa T, Kim MY, Choi BY, Orita Y, Minoda R (2019) prenatal electroporation-mediated gene transfer restores Slc26a4 knock-out mouse hearing and vestibular function. Sci Rep 9(1):1–2

Tan KS, Ong HH, Yan Y et al (2018) In vitro model of fully differentiated human nasal epithelial cells infected with rhinovirus reveals epithelium-initiated immune responses. J Infect Dis 217(6):906–915

Tang ZH, Chen JR, Zheng J, Shi HS, Ding J, Qian XD, Zhang C, Chen JL, Wang CC, Li L, Chen JZ (2016) Genetic correction of induced pluripotent stem cells from a deaf patient with MYO7A mutation results in morphologic and functional recovery of the derived hair cell-like cells. Stem Cells Transl Med 5(5):561–571

Tang Y, Xu W, Guo W et al (2017) [Primary culture of human normal epithelial cells]. J Central South Univ Med Sci 42(11):1327–1333

Tang PC, Alex AL, Nie J, Lee J, Roth AA, Booth KT, Koehler KR, Hashino E, Nelson RF (2019) Defective Tmprss3-associated hair cell degeneration in inner ear organoids. Stem Cell Rep 13(1):147–162

Tekin D, Yan D, Bademci G, Feng Y, Guo S, Foster J II, Blanton S, Tekin M, Liu X (2016) A next-generation sequencing gene panel (MiamiOtoGenes) for comprehensive analysis of deafness genes. Hear Res 333:179–184

Thomas ED, Raible DW (2019) Distinct progenitor populations mediate regeneration in the zebrafish lateral line. elife 8:e43736

Tian T, Zi X, Peng Y et al (2018) H3N2 influenza virus infection enhances oncostatin M expression in human nasal epithelium. Exp Cell Res 371(2):322–329

Tong JY, Wong A, Zhu D, Fastenberg JH, Tham T (2020) The prevalence of olfactory and gustatory dysfunction in COVID-19 patients: a systematic review and meta-analysis. Otolaryngol Head Neck Surg 163(1):3–11. https://doi.org/10.1177/0194599820926473

Topsakal V, Hilgert N, Van Dinther J, Tranebjærg L, Rendtorff ND, Zarowski A, Offeciers E, Van Camp G, Van De Heyning P (2010) Genotype-phenotype correlation for DFNA22: characterization of non-syndromic, autosomal dominant, progressive sensorineural hearing loss due to MYO6 mutations. Audiol Neurotol 15(4):211–220

Torabi R, Ranjbar R, Halaji M, Heiat M (2020) Aptamers, the bivalent agents as probes and therapies for coronavirus infections: a systematic review. Mol Cell Probes 4:101636

Tseng CC, Hu LY, Liu ME, Yang AC, Shen CC, Tsai SJ (2016) Risk of depressive disorders following sudden sensorineural hearing loss: a nationwide population-based retrospective cohort study. J Affect Disord 197:94–99

Tsukada K, Ichinose A, Miyagawa M, Mori K, Hattori M, Nishio SY, Naito Y, Kitajiri SI, Usami SI (2015) Detailed hearing and vestibular profiles in the patients with COCH mutations. Ann Otol Rhinol Laryngol 124(1_Suppl):100S–110S

Tucker AS, Dyer CJ, Romero JM, Teshima TH, Fuchs JC, Thompson H (2018) Mapping the distribution of stem/progenitor cells across the mouse middle ear during homeostasis and inflammation. Development 145(1):dev154393

Usami SI, Miyagawa M, Suzuki N, Nishio SY (2012) Genetics and presbycusis-monogenic form of age related hearing impairment caused by cdh23 mutations. J Hear Sci 2(4)

Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, Lee MK, Skvorak AB, Morton CC, Blumenfeld A (1998) Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science 279(5358):1950–1954

Vaira LA, Salzano G, Deiana G, De Riu G (2020) In Response to anosmia and ageusia: common findings in COVID-19 patients. Laryngoscope

Vélez-Ortega AC, Frolenkov GI (2019) Building and repairing the stereocilia cytoskeleton in mammalian auditory hair cells. Hear Res 376:47–57

Vermeire K, Brokx JP, Wuyts FL, Cochet E, Hofkens A, De Bodt M, Van de Heyning PH (2006) Good speech recognition and quality-of-life scores after cochlear implantation in patients with DFNA9. Otol Neurotol 27(1):44–49

Volk AE, Lang-Roth R, Yigit G, Borck G, Nuernberg G, Rosenkranz S, Nuernberg P, Kubisch C, Beutner D (2013) A novel MYO6 splice site mutation causes autosomal dominant sensorineural hearing loss type DFNA22 with a favourable outcome after cochlear implantation. Audiol Neurotol 18(3):192–199

von Bartheld CS, Hagen MM, Butowt R (2020) Prevalence of chemosensory dysfunction in COVID-19 Patients: a systematic review and meta-analysis reveals significant ethnic differences. ACS Chem Neurosci 11(19):2944–2961

Vona B, Lechno S, Hofrichter MA, Hopf S, Läßig AK, Haaf T, Keilmann A, Zechner U, Bartsch O (2016) Confirmation of PDZD7 as a nonsyndromic hearing loss gene. Ear Hear 37(4):e238–e246

Walters BJ, Cox BC (2019) Approaches for the study of epigenetic modifications in the inner ear and related tissues. Hear Res 376:69–85

Walters BJ, Coak E, Dearman J, Bailey G, Yamashita T, Kuo B, Zuo J (2017) In vivo interplay between p27Kip1, GATA3, ATOH1, and POU4F3 converts non-sensory cells to hair cells in adult mice. Cell Rep 19(2):307–320

Wang J, Tabata Y, Morimoto K (2006) Aminated gelatin microspheres as a nasal delivery system for peptide drugs: evaluation of in vitro release and in vivo insulin absorption in rats. J Control Release 113(1):31–37

Wang X, Dellamary L, Fernandez R, Harrop A, Keithley EM, Harris JP, Ye Q, Lichter J, LeBel C, Piu F (2009) Dose-dependent sustained release of dexamethasone in inner ear cochlear fluids using a novel local delivery approach. Audiol Neurotol 14(6):393–401

Wang H, Wu K, Guan J, Wang Q (2020a) Generation of a human induced pluripotent stem cell line (CPGHi001-A) from a hearing loss patient with the TMC1 p. M418K mutation. Stem Cell Res 49:101982

Wang Y, Wang Y, Chen Y, Qin Q (2020b) Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID-19) implicate special control measures. J Med Virol 92(6):568–576

Wang L, Lin ZQ, Wong A (2020c) Covid-net: a tailored deep convolutional neural network design for detection of covid-19 cases from chest x-ray images. Sci Rep 10(1):1–2

Warnecke A, Mellott AJ, Römer A, Lenarz T, Staecker H (2017) Advances in translational inner ear stem cell research. Hear Res 353:76–86

Weegerink NJ, Schraders M, Oostrik J, Huygen PL, Strom TM, Granneman S, Pennings RJ, Venselaar H, Hoefsloot LH, Elting M, Cremers CW (2011) Genotype–phenotype correlation in DFNB8/10 families with TMPRSS3 mutations. J Assoc Res Otolaryngol 12(6):753–766

Werner U, Kissel T (1995) Development of a human nasal epithelial cell culture model and its suitability for transport and metabolism studies under in vitro conditions. Pharm Res 12(4):565–571

Werner U, Kissel T (1996) In-vitro cell culture models of the nasal epithelium: a comparative histochemical investigation of their suitability for drug transport studies. Pharm Res 13(7):978–988

World Health Organization (n.d.) Addressing the rising prevalence of hearing loss

Wu WJ, Sha SH, McLaren JD, Kawamoto K, Raphael Y, Schacht J (2001) Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague–Dawley rat. Hear Res 158(1-2):165–178

Wu CC, Chen PJ, Chiu YH, Lu YC, Wu MC, Hsu CJ (2008) Prospective mutation screening of three common deafness genes in a large Taiwanese Cohort with idiopathic bilateral sensorineural hearing impairment reveals a difference in the results between families from hospitals and those from rehabilitation facilities. Audiol Neurotol 13(3):172–181

Wu CC, Liu TC, Wang SH, Hsu CJ, Wu CM (2011a) Genetic characteristics in children with cochlear implants and the corresponding auditory performance. Laryngoscope 121(6): 1287–1293

Wu CC, Hung CC, Lin SY, Hsieh WS, Tsao PN, Lee CN, Su YN, Hsu CJ (2011b) Newborn genetic screening for hearing impairment: a preliminary study at a tertiary center. PLoS One 6(7): e22314

Wu CC, Lin YH, Lu YC, Chen PJ, Yang WS, Hsu CJ, Chen PL (2013) Application of massively parallel sequencing to genetic diagnosis in multiplex families with idiopathic sensorineural hearing impairment. PLoS One 8(2):e57369

Wu CM, Ko HC, Tsou YT, Lin YH, Lin JL, Chen CK, Chen PL, Wu CC (2015) Long-term cochlear implant outcomes in children with GJB2 and SLC26A4 mutations. PLoS One 10(9): e0138575

Xia M, Ma J, Sun S, Li W, Li H (2019) The biological strategies for hearing re-establishment based on the stem/progenitor cells. Neurosci Lett 711:134406

Xiong Y, Liu Y, Cao L, Wang D, Guo M, Jiang A, Guo D, Hu W, Yang J, Tang Z, Wu H (2020) Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. Emerg Microbes Infect 9(1):761–770

Yamahara K, Asaka N, Kita T, Kishimoto I, Matsunaga M, Yamamoto N, Omori K, Nakagawa T (2019) Insulin-like growth factor 1 promotes cochlear synapse regeneration after excitotoxic trauma in vitro. Hear Res 374:5–12

Yan YJ, Li Y, Yang T, Huang Q, Wu H (2013) The effect of GJB2 and SLC26A4 gene mutations on rehabilitative outcomes in pediatric cochlear implant patients. Eur Arch Otorhinolaryngol 270(11):2865–2870

Yan D, Kannan-Sundhari A, Vishwanath S, Qing J, Mittal R, Kameswaran M, Liu XZ (2015) The genetic basis of nonsyndromic hearing loss in Indian and Pakistani populations. Genet Test Mol Biomarkers 19(9):512–527

Yan CH, Faraji F, Prajapati DP, Ostrander BT, DeConde AS (2020) Self-reported olfactory loss associates with outpatient clinical course in COVID-19. Int Forum Allergy Rhinol 10(7): 821–831

Yao XH, Li TY, He ZC, Ping YF, Liu HW, Yu SC, Mou HM, Wang LH, Zhang HR, Fu WJ, Luo T (2020) A pathological report of three COVID-19 cases by minimally invasive autopsies. Chin J Pathol 49:E009

Yin S, Huang M, Li D, Tang N (2020) Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. J Thromb Thrombolysis 3:1

Yoo JW, Kim YS, Lee SH et al (2003) Serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. Pharm Res 20(10):1690–1696

Yoshida H, Takahashi H, Kanda Y, Usami SI (2013) Long term speech perception after cochlear implant in pediatric patients with GJB2 mutations. Auris Nasus Larynx 40(5):435–439

Zhang LP, Chai YC, Yang T, Wu H (2013) Identification of novel OTOF compound heterozygous mutations by targeted next-generation sequencing in a Chinese patient with auditory neuropathy spectrum disorder. Int J Pediatr Otorhinolaryngol 77(10):1749–1752

Zhang W, Kim SM, Wang W, Cai C, Feng Y, Kong W, Lin X (2018) Cochlear gene therapy for sensorineural hearing loss: current status and major remaining hurdles for translational success. Front Mol Neurosci 11:221

Zhao Y, Wang D, Zong L, Zhao F, Guan L, Zhang P, Shi W, Lan L, Wang H, Li Q, Han B (2014) A novel DFNA36 mutation in TMC1 orthologous to the Beethoven (Bth) mouse associated with autosomal dominant hearing loss in a Chinese family. PLoS One 9(5):e97064

Zhong C, Fu Y, Pan W, Yu J, Wang J (2019) Atoh1 and other related key regulators in the development of auditory sensory epithelium in the mammalian inner ear: function and interplay. Dev Biol 446(2):133–141

Zhu J, Pang J, Ji P, Zhong Z, Li H, Li B, Zhang J, Lu J (2020) Coagulation dysfunction is associated with severity of COVID-19: a meta-analysis. J Med Virol

Ziegler CG, Allon SJ, Nyquist SK, Mbano IM, Miao VN, Tzouanas CN, Cao Y, Yousif AS, Bals J, Hauser BM, Feldman J (2020) SARS-CoV-2 receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is detected in specific cell subsets across tissues. Cell

Zipeto D, da Fonseca Palmeira J, Argañaraz GA, Argañaraz ER (2020) ACE2/ADAM17/TMPRSS2 interplay may be the main risk factor for COVID-19. Front Immunol 11

Respiratory Disorders: Contemporary Issues in 2020

8

Surinder K. Jindal and Aditya Jindal

Abstract

Respiratory system bears the major brunt of environmental insults. Respiratory infections such as pneumonias continue to threaten human health. The corona virus pandemic (COVID-19) is the most recent example of a respiratory infection threatening the human kind. Tuberculosis is one of the most ancient diseases which continue to pose as a clinical problem. Besides infections, there is a huge burden of chronic respiratory disorders in terms of morbidity and mortality. Chronic respiratory disease (CRD) is among the most common non-communicable diseases (NCDs) identified by the World Health Organization. Chronic obstructive pulmonary disease (COPD) is the third most common cause of death the world over. Respiratory allergies such as bronchial asthma, environmental, occupational and other interstitial lung diseases are other common chronic lung diseases which are increasing in incidence.

Several new diagnostic and treatment modalities have been added in our armamentarium to fight against these disorders. Besides the medical and surgical treatments, some of these interventions are non-pharmacological in nature such as the pulmonary rehabilitation and patient education programmes. Newer strategies and governmental programmes constitute other important steps to control the disease-burden.

S. K. Jindal (✉)
Postgraduate Institute of Medical Education and Research, Chandigarh, India

Jindal Clinics, Sector 20 D, Chandigarh, India

A. Jindal
Jindal Clinics, Sector 20 D, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_8

Keywords

India · Respiratory infections · Tuberculosis · Bronchial asthma · Chronic obstructive pulmonary disease · Air pollution · Pradhan Mantri Ujjwala Yojna

8.1 Introduction

Respiratory system is the gateway to our contact with outside environment through the air we breathe. All kinds of environmental air pollutants have to pass through the respiratory tract to enter the human system. Extensive exposure to air pollution caused by different noxious particles, gases and microorganisms is thus an important factor responsible for a multitude of diseases. Respiratory problems include disease conditions of respiratory tract and the lungs. There is thus an urgent need to look into the future and investigate different research issues in clinical as much as in basic sciences (Jindal 2012b).

Influenza, pneumonias and tuberculosis are some of the important respiratory infections known for centuries. Recently, the corona virus (Covid-19) pandemic which started in December 2019 in China has drawn world attention to respiratory problems in the Year 2020. Covid-19, caused by a respiratory corona virus (SARS-CoV2) has engulfed the entire world responsible for about 25 million cases and nearing a million deaths worldwide. The magnitude of the problem can largely be attributed to the air-borne spread through the respiratory system and rapid infectivity of the novel Covid-19.

Bronchial asthma and allergic rhinitis are two common respiratory allergies which affect people of all ages, especially children and young adults, all over the world. Chronic obstructive pulmonary disease (COPD) is the other most common NCD which primarily affects people of older age groups. COPD which globally is the third most common cause of death is progressively rising in incidence. Incidences of respiratory cancers has also risen significantly in the last few years. This is largely attributed to tobacco smoking and occupational exposures to different carcinogens mostly inhaled from the environment.

8.2 Historical Aspects of Respiratory Problems

History of lung disease is rather old. Breathing had been the most recognizable sign of life even in the most primitive cultures. In the ancient Vedic civilization, life was measured with the number of breaths which were believed to be fixed for every living being. Egyptians also believed that breathing was the most essential to live. It was perhaps in 300 BC that Erasistratus of Alexandria described an interplay between the air and the blood which produced the 'pneuma'—the spirit of life.

Both tuberculosis and asthma were known to the ancient Egyptian, Greek, Indian (Vedic) and Chinese civilizations of the pre-biblical periods in one or the other form (Selwyn-Brown 1938; Keers 1978). References to the presence of lung tuberculosis

can be traced to about 2000 BC in Babylonian and the Indo-Aryan cultures, for example, in the 'Rig-Veda' from the Indian continent, Pen Tsao of the Chinese *materia medica* and the Babylonian Code of Hammurabi (Nunn 1996; Cave 1939). In Vedic literature, the references are also available in the Rig-Veda, the Atharva-Veda and later in Laws of Manu.

References to asthma are recognized from descriptions of 'breathing disorders' in the ancient Egyptian, Greek, Vedic and Chinese manuscripts. '*Tamkasvasa*' mentioned in Ayurvedic literature of around 1500 BC closely resembles the modern definition of asthma (Jaggi 1961). Recognition of many other diseases can be attributed to Hippocrates during the pre-Biblical period who found the fundamentals of modern Medicine. He along with his disciples is credited with the authorship of about 70 books on different aspects of medicine. There are several references to lung infections, pneumonias and empyema—pus in the pleural cavity (Katz 1959).

Medicine in general developed faster in the post-Biblical period. Tuberculosis continued to remain the focus of attention almost throughout. Several pandemics of respiratory infections, flu and plague had threatened mankind from time to time. Some of these scourges had wiped out large segments of populations of the affected areas. It was largely during the period of Renaissance in Europe that the approach to different diseases and their treatments were more clearly understood (Udwadia 2000; Fishman 1998). Discovery of antibiotics and anti-tubercular treatments significantly helped to control infectious diseases. Application of hygienic and sterilization techniques further reduced the occurrence of infections. More recently, there is an explosion of knowledge and information. Newer diseases, tests for diagnosis and methods of treatment are continuously added. In Respiratory Medicine, the focus shifted to non-infectious diseases such as COPD, lung cancer and asthma.

History however has a peculiar way of repeating itself and teaching lessons. This is best exemplified by the resurgence of tuberculosis and occurrence of pandemics. The Flu pandemic of 1918 and now the Covid-19 have brought the world's attention back to infections (Carlos et al. 2020). The current pandemic Covid-19 has become a major global health concern. It seems obvious that man cannot get rid of infections. Although microorganisms were recognized only after the discovery of the microscope in the 1670s by Antonie van Leeuwenhoek, they had existed on the earth for billions of years—long before the birth of humans. Their adaptation to earth's environment has been more robust and holistic.

8.3 Spectrum of Respiratory Diseases

Respiratory diseases can be broadly classified under four major groups: Infections; non-infectious diseases including allergies and interstitial diseases; cancers. Tuberculosis and pneumonias are the most important groups of respiratory infections. On the other hand, there is a wide range of non-infectious lung diseases which include airway disorders (asthma and COPD), respiratory allergies, lung parenchymal disorders (Interstitial lung diseases), environmental and occupational lung diseases and neoplastic disorders. In this article, we have outlined some of the important

issues related to these diseases. In view of the deteriorating environmental standards, increasing population and urbanization, it is quite anticipated that the world will be confronted with a much larger burden of respiratory problems in the future.

8.3.1 Respiratory Infections

1. **Tuberculosis:** Tuberculosis (TB) present since the Neolithic times, has been also called as 'phthisis' or 'consumption' because it is characterized by wasting i.e. significant loss of weight (Keers 1978). It was only in 1882 that Robert Koch isolated the tubercle bacillus (Mycobacterium tuberculosis) and described its infectious cause and character. TB continues to remain a major health problem with over one-third of the global population being infected with TB bacteria. The disease was declared a global emergency by the WHO in 1993. It continues to kill millions of people worldwide, particularly so in the low- and middle-income countries. India is the largest TB burden country in the world.
   Tuberculosis caused by Mycobacterium tuberculosis most commonly involves the lungs but can affect any other organ. It is relatively insidious in onset and slow in progression but it is not uncommon for TB to present with acute manifestations sometimes posing as an emergency situation which may well be life-threatening. Tuberculosis of lungs and/or other organs may also complicate a medical or surgical emergency not directly related to tuberculosis. Approach to diagnose and treatment differ in different scenarios.

   The occurrence as well as the management of different types of TB may also depend upon the presence of risk factors and co-morbid conditions. There are higher chances of TB in the presence of diseases such as diabetes mellitus, malnutrition, human immunodeficiency virus (HIV) infection, other immunodeficiency states, pneumoconiosis such as silicosis, and concomitant respiratory diseases such as asthma, COPD, interstitial lung disease (ILD), and lung cancer. Children, elderly and those who are heavy smokers and/or alcohol abusers are also more vulnerable to develop complications. TB commonly presents with cough, phlegm, fever, other constitutional symptoms and weight loss. Occasionally, it is responsible for emergency situations of massive haemoptysis, spontaneous pneumothorax and acute respiratory distress syndrome. Similarly, extra-pulmonary tuberculosis, especially of central and cardiovascular systems may present with emergency conditions.

   Since the last decade of the twentieth century, dual infection with TB along with HIV infection has emerged as a major global health challenge (Mayer and Hamilton 2010). The problem of multi-drug resistance has further complicated the diagnosis and management issues. Several technical advances have been also made to achieve an early diagnosis and presence of drug resistance. Discovery and implementation of at-site, rapid molecular kit-based test for the diagnosis of presence and resistant organisms have proved as a game changer (Steingart et al. 2013). New drugs have been added to the management regimens for the first time in the last 3–4 decades. Several countries have designed National Programmes to

control the disease. In India, the Revised National TB Control Programme has been augmented to achieve elimination by 2025 and renamed as National TB Elimination Program (NTEP) (National Strategic Plan for Tuberculosis Elimination 2017).

It is quite an ambitious target to achieve NTEP goal of elimination of TB by 2025 (defined as incidence of less than 20 cases per million population). But there is a strong political and administrative commitment. Several new strategies have been planned under NTEP which include the engagement of the private sector, avoiding loss of patients from the care cascade, 'active case finding' among high-risk populations, management of latent and of drug-resistant TB. There is a global focus on India to learn from these experiences. TB is perhaps one of the few examples of an ancient disease which is likely to fade out in the foreseeable future.

2. **Non-tuberculous lung infections:** There are a large number of non-tubercular infections caused by various microorganisms such as the bacteria, other mycobacteria, viruses, fungi and parasites which vary in their spectrum and severity. Incidence of opportunistic infections has significantly increased mostly due to an increase in the use of immunosuppressive treatments for various diseases including organ transplantation. Technological developments of molecular diagnosis and DNA finger-printing have made it possible to identify the individual organism responsible for these otherwise obscure infections. The zoonotic origin of some of these microbes such as the Swine flu, Bird Flu and Covid-19 infections (MERS 1 and MERS 2) pose even a greater threat of large epidemics. The most recent SARS-CoV2 infection has clearly shown that the microorganisms are invincible in spite of the continued discovery of newer antibiotics and other anti-microbial drugs.

Covid-19 infection Covid-19 infection starts with the involvement of upper respiratory tract with flu-like symptoms such as fever, headache and cough; myalgias, loss of smell, anorexia, fatigue and abdominal discomfort (Pascarella et al. 2020). The infection may involve the lungs generally after about 5 days. Respiratory involvement can be categorized as severe when there is occurrence of pneumonia. Severe illness occurs in about 15% of patients while about 5% patients develop severe respiratory insufficiency. The disease carries higher mortality in males, elderly and those with underlying co-morbid conditions. The virus is not only highly infective, but also shows rapid progression. There are a number of issues related to its pathogenicity, immunogenicity, treatment and prevention which remain to be answered. It is important to practice public health interventions such as wearing of face-masks, maintaining a safe social distance in public places, repeated cleaning of hands and avoiding unnecessary travel to contain the spread of the pandemic. Simple cloth cover for face while visiting a crowded surrounding is a fairly sustainable practice (Jindal et al. 2020a). It is quite likely however that we have to live with this virus for quite some time in the future.

Anti-microbial agents and drug resistance Emergence of new infections requires newer anti-microbial agents for an adequate control of infection. Incidentally, the discovery of newer drugs is not as fast as the recognition of infecting microbes. As an example, even though the causative corona-virus responsible for the pandemic Covid-19 was detected and appropriately codified when the disease was recognized, the world is still struggling to find an effective anti-viral drug for its treatment. The situation is even worse with drug-resistant organisms. It has in fact become difficult to treat a large number of multi-drug-resistant infections which are increasing in numbers and severity. Antimicrobial drug resistance is a serious global challenge (WHO 2020). The problem is often serious in critical care units with life-threatening, drug-resistant pneumonias and septicaemia. Widespread and inappropriate use of antibiotics adds to the problem of drug resistance. It has been considered important to introduce and implement 'antibiotic-stewardship' in hospitals to prevent misuse of antibiotics (Shira Doron and Davidson 2011). It is also important to prohibit 'on the shelf' sale of antibiotics frequently to patients with self-limiting infections.

Vaccination Vaccination against bacterial and viral infections is an important consideration to achieve a reduction in overall morbidity and mortality from respiratory disease. It is not at all surprising to point to the anxiety with which the whole world is waiting for the anti-Covid vaccination.

Vaccination for measles, mumps and diphtheria, now practiced for several decades all over the world, has been responsible for a significant and appreciable fall in childhood infections. Small pox and polio have been eliminated largely due to the vaccination policy. Use of anti-TB (BCG) vaccination has been somewhat debatable. It is however found to be useful especially for prevention of serious forms of tuberculosis in children and continues to be routinely used at birth in India.

Anti-influenza and anti-pneumococcal vaccines are now recommended in adults for prevention of different types and pneumococcal diseases. These vaccinations are found as a useful cost-effective method for prevention especially in the elderly, patients with chronic respiratory diseases (CRDs) and other co-morbidities (Leidner et al. 2019). Vaccinations are helpful to reduce the occurrence of infection as well as the disease complications and worsening. They indirectly help to reduce hospitalization and health-care costs.

8.3.2 Non-infectious Respiratory Problems

Chronic, non-communicable diseases (NCDs) have posed a major health challenge in the twenty-first century. The issue was taken up in the United Nations General Assembly in September 2018 in its third High-level Meeting on the prevention and control of NCDs (WHO 2018). Various strategies and steps were suggested after a comprehensive review of the global and national scenario focussing on chronic heart and lung diseases, cancers and diabetes. Amongst various NCDs, CRD is one of the important issues of concern. As per Global Burden of Disease Study, asthma and COPD are the two most common CRDs which constitute 8.3% of the overall burden

of chronic diseases (GBDS 2017). COPD remains the third leading cause of mortality worldwide, responsible for 5.5% of deaths annually (WHO 2018). CRD is an important cause of impairment of patients' quality of life, ability, and productivity. It is also responsible for an increased economic burden for both the patient and the health-care infrastructure. It is important to mention that several risk factors (tobacco smoking, physical inactivity, obesity and nutrition) are common for most of the NCDs, including the CRDs. Control of the common risk factors is of paramount importance to reduce the overall burden of NCDs including the chronic respiratory problems.

8.3.2.1 Bronchial Asthma and Respiratory Allergies

Bronchial asthma is a chronic airway disease which results from a combination of aetiological factors, both genetic and environmental in origin. It is labelled as an allergic disorder since it frequently manifests in genetically predisposed people with atopic sensitization. The episodes are commonly triggered following respiratory infections or due to exposure to airborne allergens such as the pollens, dusts and smoke. The disease is characterized by episodic airway obstruction which is frequently reversible, at least partially so. Asthma often starts during childhood but may appear at any time during life. It is more commonly seen in patients with personal and/or family history of the presence of allergic disorders.

As per global estimates in 2016, asthma accounted for a global burden of over 300 million people with variable prevalence across countries. It has been also estimated that the number is likely to exceed 400 million by the year 2025 (GBDS 2017). The disease is now reported from low- and middle-income countries with almost the same frequency as from high-income countries. As per population surveys, there is about 3–5% prevalence of asthma in India. Asthma is also an important cause of high economic burden. It is an important cause of school absenteeism among children, interferes with daily activities and work performance of adults. Asthma impairs the quality of life as well as increases hospitalizations and health-care costs. Asthma is also reported as a cause of death in about half a million patients every year (WHO 2018).

Inhalational therapy Even though the inhalation of different herbal vapours and smokes is old, it has become popular as a mode of management of respiratory disease in the last two hundred years or so. The modern metered dose devices and other sophisticated inhalers have replaced the earthen and metal pots which were used earlier. Inhalational therapy with corticosteroids and bronchodilators constitutes the core of effective anti-asthma management which is now available. Newer and effective drugs and devices continue to flood the market without any major advantage over one another (Dalby and Suman 2003). Commonly, there are two types of medical inhalers: (1) dry powder inhalers and (2) metered dose liquid inhalers. They have their own advantages and disadvantages and therefore used according to patient's preferences and ease of administration. Nebulization of different drugs is also a type of liquid inhalation used in emergency situations or when MDIs are difficult to administer (e.g. in children, elderly, very sick,

semi-conscious or unconscious patients). Future developments in inhalational technology are likely to significantly improve the effective and safe drug delivery to the airways (Biddiscombe and Usmani 2018).

Phenotyping and individualized therapy There is a large degree of heterogeneity of clinical and patho-physiological characteristics of asthma which suggest that asthma is not a single disease but presents with different phenotypes (Papi et al. 2017). Traditionally considered as an atopic disorder, it is now believed that less than 50% of cases have eosinophilic airway inflammation. Severe and persistent asthma may in fact be present in non-eosinophilic phenotypes. Asthma phenotyping is usually based on clinical, trigger-related, demographic and pathological features. A multi-dimensional approach is now advocated using a latent class analysis with the help of statistical methods of cluster analysis. Some people also define allergic multi-morbidity phenotypes based on eczema, rhinitis and asthma.

Phenotyping helps to identify different treatment modalities for different patients—individualized or target therapy (Fajt and Wenzel 2015). Better control can be achieved with this approach for uncontrolled or poorly-controlled, severe asthma. There is an increase in incidence of severe asthma. This has brought the issue of different disease phenotypes and precision medicine into sharp focus. A number of biological agents which consist of mono-clonal antibodies which target a particular cytokine in the pathogenesis cycle of asthma are available. This approach has significantly impacted the disease management. The approach of phenotyping and individualized therapy is now being used for several other diseases including COPD and lung cancer. We are likely to see individualized therapy with different biological agents targeted to antagonize the actions of different mediators and cytokines responsible for inflammation.

8.3.2.2 Chronic Obstructive Pulmonary Disease

COPD, characterized by irreversible or partially reversible airflow limitation, is relentlessly progressive and fatal in nature (GOLD 2020). It is a major cause of disability and the third most common cause of death worldwide (GBDS 2017; WHO 2018; GOLD 2020). The disease prevalence in India has increased in an epidemic proportion in the last few decades with an overall prevalence of about 3% (GOLD 2020; Jindal et al. 2012; Jindal 2012a, b). The number of patients has shown an increase in prevalence from 3·3% in 1990 to 4·2% in 2016 (India State-Level Disease Burden Initiative 2018). Tobacco smoking is the most important risk factor responsible for COPD in over 80% of male patients. Environmental and occupational exposures as well as household air pollution (HAP) are other important risk factors, especially among female and non-smoker male patients. COPD causes cough, expectoration and breathlessness, frequently with acute exacerbations. A large number of co-morbidities such as cardiovascular disease, diabetes, muscle wasting, osteoporosis and bone loss, depression and other neuro-psychiatric are also common in COPD patients.

COPD management consists of maintenance, pharmacological treatment for symptomatic relief and prevention of complications. Inhalational and oral

bronchodilators constitute the mainstay of pharmacological treatment. Patient education can also play an important role to improve the quality of life and response to exacerbations, ability to cope with illness and the understanding of terminal care and ability to make decisions. Long-term domiciliary oxygen therapy, pulmonary rehabilitation, vaccination and health education are important strategies to achieve the long-term goals. Smoking cessation is the most important step to prolong survival in COPD. Smoking cessation methods consist of self-help quitting plan, professional counselling and pharmacological smoking aversion therapy under the guidance of a physician. Risk factor reduction will also include the removal or minimization of occupational exposures as well as of household and outdoor air pollution.

Biomass smoke-exposed COPD Almost half of the global population use biomass fuels such as dried cattle-dung, forest residue and wood for domestic combustion for cooking and heating. While over 2.8 billion people use simple stoves for this purpose, there are 1.2 billion people who use simple kerosene lamps to light their homes (Bonjour et al. 2013). Burning of these fuels is an important source of household air pollution. Both women and children are therefore chronically exposed to the smoke from these fuels. This is an important cause of increased respiratory infections and impaired respiratory health in women and children of the low- and middle-income countries. In women, this is responsible for ill health and CRD such as COPD and chronic bronchitis. Continued exposure leads to progressive disability and respiratory failure (Mortimer et al. 2012; Gordon et al. 2014).

Biomass smoke-exposed COPD is now considered as a different phenotype than the smoker COPD (Ji et al. 2018; Jindal 2018). There is predominant involvement of small airways with more frequent symptomatic worsening and acute exacerbations. There is a higher frequency of acute emergency visits and hospitalization responsible for greater utilization of health-care resources (Jindal et al. 2020b, c). These patients are also likely to respond better to anti-inflammatory treatment with inhalational corticosteroids. The problem is significantly avoidable with the use of cleaner fuels at home. Massive and consistent efforts are required to achieve any meaningful health benefits in view of the huge magnitude of the problem. *Pradhan Mantri Ujjwala Yojna* (PMUY) introduced by the Government of India is one such example. PMUY coupled with smoking-cessation strategies are essential to reduce the COPD burden (Jindal et al. 2020b, c).

Pradhan Mantri Ujjwala Yojna PMUY aimed to replace biomass fuels with cleaner cooking gas, LPG. The scheme to distribute 50 million free/subsidized connections for cleaner fuel to women was introduced by the Prime Minister of India in 2016 (PMUY 2016). The scope of the scheme was expanded to include 80 million poor households. Several other initiatives were also included during the plan-implementation. There are several barriers which require additional inputs to change the behavioural practices amongst rural populations. It will take time before one can assess the health benefits. But it remains one of the largest interventions to achieve better environmental and health targets.

8.3.2.3 Interstitial Lung Disease

Interstitial Lung Disease (ILD) is a group of over 300 different conditions of diverse aetiologies which involve the lung parenchyma (Travis et al. 2013). The group includes diseases which occur secondarily to other systemic conditions, allergic and hypersensitivity disorders, environmental and occupational exposures and iatrogenic conditions (Travis et al. 2013; Singh et al. 2017). There is a large group of idiopathic conditions which account for 'true' or primary ILDs recognized as Idiopathic Interstitial Pneumonia (IIP). ILD, particularly the IIP is progressive in nature ultimately leading to damage of the lung tissue, pulmonary fibrosis and shrinkage of lungs. Idiopathic pulmonary fibrosis (IPF) which is relentlessly progressive is an important cause of severe respiratory disability and death. Both the recognition and the morbidity of IPF have significantly risen during the last 2–3 decades including in India (Dhooria et al. 2018).

Treatment of ILD is highly unsatisfactory but for the treatment of the underlying illness in case of a secondary cause. IPF treatment remains a challenge in spite of some significant advances made in our understanding of the pathogenesis and treatment. IPF, earlier believed as an inflammatory condition, is now recognized as characterized with recurrent episodes of lung damage and aberrant repair, neo-vascularization and apoptosis. Several of these pathological processes are almost akin to those of a neoplastic illness. We now have two anti-fibrotic drugs which are available in the market. Both the drugs however are useful only to slow down the progressive decline in lung function (Dhooria et al. 2018; Ogura et al. 2015; Vancheri et al. 2018). There are several other drugs in the pipeline which are being tested for treatment. Lung transplantation is done for the severely and irreversibly damaged and fibrosed lungs (Kistler et al. 2014; ten Klooster et al. 2015). Novel approaches are required to stop the progression of and/or reverse fibrosis.

Ambient air pollution, Environmental and occupational disorders A large number of environmental and occupational dusts which pollute the ambient air in living and/or working surroundings are responsible for airways and lung-parenchymal disorders. Ambient air pollution due to industrial and vehicular exhausts is a major cause of worry in the developing countries (Boogaard et al. 2019; Khilnani and Tiwari 2018). On the one hand, there is an increased need and demand for industrialization and urbanization to void the ever-increasing economic pressures. On the other hand, both these factors are responsible for increased pollution and decreased air quality. Air pollution adversely affects the general well-being and more so the respiratory health (Hoek et al. 2013). Air pollution is singularly responsible for increase in acute respiratory infections as well as CRDs.

Besides the general air pollution and its adverse effects on the lungs, there are other types of environmental and occupational air pollutants which cause specific lung diseases such as hypersensitivity pneumonias (HP), pneumoconioses and reactive airway syndromes. HP is commonly attributed to exposure to organic dusts in a previously sensitized individual. There are numerous examples depending on the type of exposure. HPs like Farmer's lung, Psittacosis, Bird-fancier's lung, Byssinosis, and Baggasosis are some common and important causes of ILD and

pulmonary fibrosis. Chronic HP was singularly the most common cause of pulmonary fibrosis in India (Singh et al. 2017).

Pneumoconioses such as silicosis, anthracosis and asbestosis occur due to deposition of inorganic dusts due to work exposure among miners, factory and foundry workers (Cullinan and Reid 2013). Silicosis is an important deposition lung disease responsible for progressive lung fibrosis. The prevalence in Western countries has diminished because of extensive adoption of preventive measures but has not disappeared (Barnes et al. 2019). The problem is quite huge in India so that the Supreme Court of India had to intervene in a landmark judgement and asks the National Human Rights Commission to take up the specific and confirmed cases of persons who are suffering from silicosis (Jindal 2013; Supreme Court of India 2009).

Asbestosis due to inhalation and deposition of asbestos fibres in the lungs is another important cause of ILD and lung fibrosis (Lazarus and Philip 2011). Asbestos is also an important precursor for lung cancer development. Its use has been banned in several countries. Exposure to toxic gases and fumes can produce different types of reactive airways syndromes and lung damage. When massive, such an exposure can also be fatal. The Bhopal Gas disaster due to industrial leakage of toxic gas (Methyl Iso-Cyanate) from pesticide plant in December 1984 is the most glaring example of such a tragedy (Mishra et al. 2009).

Lung transplantation Lung transplantation is the only option of treatment for irreversibly damaged lungs due to any disease such as the ILD, COPD, cystic fibrosis, bronchiectasis and others (Kistler et al. 2014; ten Klooster et al. 2015; Young and Dilling 2019). There are already a few examples of transplantation being done in patients with COVID-19 induced lung fibrosis (Cypel and Keshavjee 2020). People have tried different types of transplantation of a single, double-lung or the whole heart-lung block. Partial transplantation of a part of the lung has been also tried. There are some major barriers to the availability of this treatment which include the relative lack of resources, expertise and most importantly of the donor lungs. Postoperative care, rehabilitation and management of complications such as the rejection and infection are other important issues. A state-wide or preferably a country-wide network and maintenance of a Registry are essential for optimum utilization of resources and availability of treatment.

8.3.3 Respiratory Cancers

Respiratory cancers have seen a major increase in the modern times (Nasim et al. 2019). Lung cancer is the most common cancer among men and second most common among women. Tobacco smoking is the most common cause of respiratory cancers but it is also seen in non-smokers (Rivera and Wakelee 2016). Increased female incidence is largely attributed to an increase in the smoking habit among women in Western countries. Exposures to radon-daughters, asbestos, nickel, chromium and mercury are also reported to be carcinogenic. There are a few reports on exposure to biomass smoke especially from coke being also carcinogenic. Diagnosis

of lung cancer is made from a conglomeration of clinical, radiological and cyto-histopathological findings.

Significant advances have taken place in our knowledge of the aetio-pathogenesis and pathology of lung cancer (Nasim et al. 2019). Most recent classification of lung cancer identifies different types based on pathological and immuno-histo-chemistry characteristics. It is classified into two major categories (small-cell and non-small cell lung cancer) for purpose of treatment. Lung cancer staging is important to decide the type of treatment. Surgery is the best option for Stage 1 and 2 A cancers. Treatment of Stage 3 and 4 cancers is done with chemo- and/or radiation therapy. Prognosis of lung cancer remains poor unless curative surgery is possible. Therefore, early diagnosis is the key to a successful treatment.

8.4 Summary

Respiratory system is exposed to environmental insults of all kinds making it prone to suffer both infective and non-infective respiratory diseases which pose a dual health challenge in the twenty-first century. While many other chronic illnesses have either reached plateau or started declining, chronic respiratory illnesses continue to increase in incidence. There is an obvious need to reduce risk factors such as tobacco smoking, environmental air pollution and biomass fuel combustion. All these risk factors are significantly modifiable. Another modifiable factor of importance is the treatment-seeking behaviour which significantly influences the progression and outcome of any illness. This is of special significance for respiratory conditions where maintenance pharmacotherapy as well as non-pharmacological measures have a direct bearing on respiratory health. There is also a need to broaden the scope of surveillance activities. This is especially so in a developing country like India where the data on disease prevalence and progression are limited in view of the huge geographical, cultural, social, religious, political and economic diversities. Continued surveillance and monitoring are also required to help the policy planners as well as the health administrators.

References

Barnes H, Goh NSL, Leong TL, Hoy R (2019) Silica-associated lung disease: an old-world exposure in modern industries. Respirology 24(12):1165–1175

Biddiscombe MF, Usmani OS (2018) Is there room for further innovation in inhaled therapy for airways disease? Breathe (Sheff) 14(3):216–224

Bonjour S, Adair-Rohani H, Wolf J et al (2013) Solid fuel use for household cooking: country and regional estimates for 1980–2010. Environ Health Perspect 121:784–790

Boogaard H, Walker K, Cohen AJ (2019) Air pollution: the emergence of a major global health risk factor. Int Health 11(6):417–421

Carlos WG, Dela Cruz CS, Cao B, Pasnick S, Jamil S (2020) Novel Wuhan (2019-nCoV) coronavirus. Am J Respir Crit Care Med 201:P7–P8

Cave AJE (1939) The evidence for the incidence of tuberculosis in ancient Egypt. Br J Tuberc 33: 142

Cullinan P, Reid P (2013) Pneumoconiosis. Prim Care Respir J 22(2):249–252
Cypel M, Keshavjee S (2020) When to consider lung transplantation for COVID-19. Lancet Respir Med S2213-2600(20)30393-3
Dalby R, Suman J (2003) Inhalation therapy: technological milestones in asthma treatment. Adv Drug Deliv Rev 55:779–791
Dhooria S, Agarwal R, Dhar R, Jindal A, Madan K, Aggarwal AN et al (2018) Consensus statement for the diagnosis and treatment of idiopathic pulmonary fibrosis in resource constrained settings. Indian J Chest Dis Allied Sci 60:93–121
Fajt ML, Wenzel SE (2015) Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. J Allergy Clin Immunol 135(2): 299–310
Fishman AP (1998) Milestones in the history of pulmonary medicine. In: Fishman's pulmonary diseases and disorders, 3rd edn. McGraw-Hill, New York
GBDS (2017) Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 390:1211–1259
Global Initiative for Chronic Obstructive Lung Disease (2020) Global strategy for the diagnosis, management and prevention of chronic obstructive lung disease (2020 report). https://goldcopd.org. Accessed 20 Sept 2020
Gordon SB, Bruce NG, Grigg J et al (2014) Respiratory risks from household air pollution in low and middle income countries. Lancet Respir Med 2:823–860
Hoek G, Krishnan RM, Beelen R, Peters A, Ostro B, Brunekreef B et al (2013) Long-term air pollution exposure and cardio-respiratory mortality: a review. Environ Health 12(1):43
India State-Level Disease Burden Initiative CRD Collaborators (2018) The burden of chronic respiratory diseases and their heterogeneity across the states of India: the Global Burden of Disease Study 1990–2016. Lancet Glob Health 6:e1363–e1374
Jaggi OP (1961) Chest diseases in ancient Hindu medicine. Indian J Chest Dis 3:124–128
Ji W, Lim MN, Bak SH et al (2018) Differences in chronic obstructive pulmonary disease phenotypes between non-smokers and smokers. Clin Respir J 12(2):666–673
Jindal SK (2012a) COPD: the unrecognized epidemic in India. J Assoc Physicians India 60 (Suppl):14–16
Jindal SK (2012b) Research in clinical sciences. Indian J Chest Dis Allied Sci 54:175–182
Jindal SK (2013) Silicosis in India: past and present. Curr Opin Pulm Med 19:163–168
Jindal SK (2018) Chronic obstructive pulmonary disease in non-smokers—is it a different phenotype? Indian J Med Res 147(4):337–339
Jindal SK, Aggarwal AN, Gupta D, Agarwal R, Kumar R, Kaur T, Chaudhry K, Shah B (2012) Indian study on epidemiology of asthma, respiratory symptoms and chronic bronchitis in adults (INSEARCH). Int J Tuberc Lung Dis 16(9):1270–1277
Jindal SK, Aggarwal AN, Christopher DJ, Dhar R, Jindal A (2020a) Cloth face covers—a sustainable measure to mitigate COVID-19. Int J Tuberc Lung Dis. https://doi.org/10.5588/ijtld.20.0220
Jindal SK, Aggarwal AN, Jindal A et al (2020b) Exacerbation rates are higher in chronic obstructive pulmonary disease in non-smokers in India. Int J Tuberc Lung Dis 24(12):1272–1278
Jindal SK, Aggarwal AN, Jindal A (2020c) Household air pollution in India and respiratory diseases: current status and future directions. Curr Opin Pulm Med 26(2):128–134
Katz AM (1959) Hippocrates and the plane tree on the island of Cos. Arch Intern Med 104:653–657
Keers RY (1978) Pulmonary tuberculosis. A journey down the centuries. Bailliere-Tindall, London
Khilnani GC, Tiwari P (2018) Air pollution in India and related adverse respiratory health effects: past, present, and future directions. Curr Opin Pulm Med 24(2):108–116
Kistler KD, Nalysnyk L, Rotella P, Esser D (2014) Lung transplantation in idiopathic pulmonary fibrosis: a systematic review of the literature. BMC Pulm Med 14:139
Lazarus AA, Philip A (2011) Asbestosis. Dis Mon 57(1):14–26

Leidner AJ, Murthy N, Chesson HW, Biggerstaff M, Stoecker C, Harris AM, Acosta A, Dooling K, Bridges CB (2019) Cost-effectiveness of adult vaccinations: a systematic review. Vaccine 37(2):226–234

Mayer K, Hamilton C (2010) Synergistic pandemics: confronting the global HIV and tuberculosis epidemics. Clin Infect Dis 50 Suppl 3:S67–S70. https://doi.org/10.1086/651475

Mishra PK, Samarth RM, Pathak N, Jain SK, Banerjee S, Maudar KK (2009) Bhopal Gas Tragedy: review of clinical and experimental findings after 25 years. Int J Occup Med Environ Health 22(3):193–202

Mortimer K, Gordon SB, Jindal SK, Accinelli RA, Balmes J, Martin WJ II (2012) Household air pollution is a major avoidable risk-factor for cardiopulmonary disease. Chest 142:1308–1315

Nasim F, Sabath BF, Eapen GA (2019) Lung cancer. Med Clin North Am 103(3):463–473

National Strategic Plan for Tuberculosis Elimination (2017–2025) Central TB Division, Ministry of Health and Family Welfare, Government of India. www.tbcindia.nic.in. Accessed 15 Sept 2020

Nunn JF (1996) Ancient Egyptian medicine. University of Oklahoma Press, Norman

Ogura T, Taniguchi H, Azuma A, Inoue Y, Kondoh Y, Hasegawa Y et al (2015) Safety and pharmacokinetics of nintedanib and pirfenidone in idiopathic pulmonary fibrosis. Eur Respir J 45:1382–1392

Papi A, Saetta M, Fabbri L (2017) Severe asthma: phenotyping to endotyping or vice versa? Eur Respir J 49:1700053

Pascarella G, Strumia A, Piliego C, Bruno F, Del Buono R, Costa F, Scarlata S, Agrò FE (2020) COVID-19 diagnosis and management: a comprehensive review. J Intern Med 288(2):192–206

PMUY (Pradhan Mantri UjjwallaYojna) (2016) Ministry of Petroleum & Natural Gas, Govt of India. https://pmuy.gov.in/about.html. Accessed 18 Sept 2020

Rivera GA, Wakelee H (2016) Lung cancer in never smokers. Adv Exp Med Biol 893:43–57

Selwyn-Brown A (1938) The physician throughout the ages, vol I. Capehart-Brown Co., Inc. Publishers, Times Building, New York

Shira Doron S, Davidson LE (2011) Antimicrobial stewardship. Mayo Clin Proc 86(11):1113–1123

Singh S, Collins BF, Sharma BB, Joshi JM, Talwar D, Katiyar S et al (2017) Interstitial lung disease in India. Results of a prospective registry. Am J Respir Crit Care Med 195(6):801–813

Steingart KR, Sohn H, Schiller I, Kloda LA, Dendukuri N (2013) Xpert® MTB/Rif assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev 1: CD009593

Supreme Court of India (2009) Writ petition (Civil) No. 110 of 2006. No. 318926, dated 3.5.2009

ten Klooster L, Nossent GD, Kwakkel-van Erp JM, van Kessel DA, Oudijk EJ, van de Graaf EA et al (2015) Ten-year survival in patients with idiopathic pulmonary fibrosis after lung transplantation. Lung 193:919–926

Travis WD, Costabel U, Hansell DM, King TE Jr, Lynch DA, Nicholson AG et al (2013) An official American Thoracic Society/European Respiratory Society statement: update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. Am J Respir Crit Care Med 188:733–748

Udwadia FE (2000) Man and medicine—a history. Oxford University Press, New Delhi

Vancheri C, Kreuter M, Richeldi L, Ryerson CJ, Valeyre D, Grutters JC et al (2018) Nintedanib with add-on pirfenidone in idiopathic pulmonary fibrosis: results of the INJOURNEY trial. Am J Respir Crit Care Med 197:356–363

WHO (2018) Third United Nations High-level Meeting on NCDs. Non-communicable diseases and their risk factors. https://www.who.int/ncds/governance/third-un-meeting. Accessed 20 Sept 2020

World Health Organization (2020) Antimicrobial resistance. Key facts. https://www.who.int. Accessed 22 Sept 2020

Young KA, Dilling DF (2019) The future of lung transplantation. Chest 155(3):465–473

9 Discovery of Hepatitis Viruses and Two Nobel Prizes: A Tale of Keen Observations, Serendipity, Collaborative Research, Astute Interpretations and Game-Changing Innovations

Arka De and Yogesh K. Chawla

Abstract

Chronic viral hepatitis, caused by hepatitis B and hepatitis C viruses, is one of the most common causes of end-stage liver disease and liver cancer in the world, and accounts for almost 4000 deaths per day. Fundamental research in chronic viral hepatitis has led to dramatic changes in its diagnosis, prevention and management. Indeed, the scientists credited with the discovery of hepatitis B (Baruch Blumberg) and hepatitis C (Harvey J. Alter, Michael Houghton and Charles M. Rice) were honoured with the Nobel Prize in 1976 and 2020, respectively. The story of the unravelling of the mysteries behind "serum hepatitis" and the establishment of its viral aetiology is an incredible testament to the remarkable accomplishments of modern biomedical research. In this chapter, we embark on a journey into the discovery of hepatitis B and C viruses, discuss the work of the Nobel laureates and throw light on the work of the unsung heroes without whose contributions, the discoveries may never have seen the light of day.

Keywords

Hepatitis B discovery · Hepatitis C discovery · Nobel Prize hepatitis

A. De
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Y. K. Chawla (✉)
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Kalinga Institute of Medical Sciences (KIMS), Bhubaneshwar, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_9

9.1 Introduction

Chronic viral hepatitis, caused by Hepatitis B and Hepatitis C viruses, is one of the most common causes of end-stage liver disease and liver cancer in the world, and accounts for almost 4000 deaths per day (Cooke et al. 2019). In fact, mortality from chronic viral hepatitis is comparable to that of tuberculosis, malaria and HIV. As per the Global Hepatitis Report of the World Health Organisation (WHO), the global burden of Hepatitis B and Hepatitis C is estimated to be 257 million and 71 million, respectively (WHO 2017). In India, approximately 50 million individuals are estimated to have chronic Hepatitis B. Higher prevalence of Hepatitis B has been noted in several tribal pockets in India (Tandon et al. 1996). The prevalence of Hepatitis C in India is estimated to be 0.5–1% with regions of higher prevalence in the North East and Punjab (Sood et al. 2018; Goel et al. 2019). The prevalence of both viruses is much higher in high-risk groups like intravenous drug users.

Fundamental research in chronic viral hepatitis has led to dramatic changes in its diagnosis, prevention and management. It is thus apt that the scientists credited with the discovery of both these viruses have been honoured with the Nobel Prize which was ordained by Sir Alfred Nobel for being awarded to those whose work "has conferred the greatest benefit to humankind". In 1976, Baruch Blumberg received the Nobel Prize in Medicine for his discovery of Hepatitis B and in 2020, Harvey J. Alter, Michael Houghton and Charles M. Rice were awarded the same for their contributions in discovering Hepatitis C. The work of these men revolutionised fields of virology, immunology and hepatology and is a remarkable story of the triumph of passion, determination, and innovative, multifaceted research. In this chapter, we will embark on a journey into the discovery of Hepatitis B and C viruses. While discussing the work of the Nobel laureates, we will also be throwing light on the work of the unsung heroes without whose contributions, the discoveries may never have seen the light of day.

9.2 Viral Hepatitis: A Brief Primer

The human hepatitis virus includes five viruses (A-E). Hepatitis A and E are transmitted by the faeco-oral route and present acutely with jaundice. Unremarkable recovery is usually the rule although sometimes acute liver failure and even death can occur. Hepatitis B and C viruses are transmitted parenterally by contaminated injections or blood. While acute presentations can occur with Hepatitis B and rarely with Hepatitis C, the bigger concern with these two viruses is the establishment of chronic infection with propensity to progress to end-stage liver disease and hepatocellular cancer. Hepatitis D is only seen in the context of chronic Hepatitis B infection and is extremely rare in India.

9.3 Prelude

9.3.1 Early Human Understanding of Hepatitis

The earliest description of jaundice can be seen in the Babylonian Talmud (dating back to around 4500 BC) and in ancient Ayurvedic and Chinese texts. The Greek physician, Hippocrates (460–370 BC) described an outbreak of jaundice in Thassos (Wong et al. 2015; Khuroo and Sofi 2020; Trepo 2014). Medieval Europe was afflicted by repeated epidemic outbreaks of jaundice because of the poor standards of hygiene and overcrowding. The communicable nature of the disease was widely suspected and an early evidence of human intervention to stop the spread of the disease can be seen in a letter written by Pope Zacharis to the Archbishop of Mainz in 751 AD. The Pope requested the Archbishop to delay communion to people with jaundice till the healthy individuals have been attended. Intriguingly, he also asked for the horses to be buried.

9.3.2 Jaundice and the Military

Epidemics and outbreaks of jaundice were a frequent scourge in military camps and during Wars in the seventeenth–nineteenth century (Wong et al. 2015). This "campaign jaundice" was known as "Soldatengelbschut" in German and "jauniesse des camps" by the French. In the late eighteenth century, the ravishing army of Napoleon suffered serious setbacks in Egypt with hundreds and thousands falling ill. More than 70,000 cases of jaundice were reported during the American Civil War. The Franco-Prussian War and Boer War similarly witnessed outbreaks of jaundice which significantly dampened troop motivation (Schmidt 1999).

9.3.3 Early Scientific Work on the Etiology of Jaundice

Precious little was known about the origin and causes of jaundice till the nineteenth century. Various reasons were attributed including bad air, foul mood, bad water, dietary changes and small "germs". Virchow, the Father of Pathology, proposed the "cattarhal" mechanism in which jaundice resulted from blockage of bile ducts by mucus (Gruber and Virchow 1865). His theory was later disproved by Murchison in 1874 who demonstrated the absence of biliary obstruction in a series of autopsies among patients who had expired during outbreaks of jaundice in Essen, London and Rotterdam (Murchison et al. 1886). In hindsight, most of the outbreaks that we have previously described were probably caused by the faeco-orally transmitted Hepatitis A and E viruses.

9.3.4 The Vaccination Era

With the dawn of the era of vaccines, transfusion hepatitis or serum hepatitis came into attention. The link was first identified by the keen observations of Lurmen while investigating an outbreak of jaundice in a Bremen dock in 1883–1884. He found that almost 15% of the 1289 individuals who were vaccinated using a particular batch of small pox vaccine developed jaundice within 6 months while none of the 500 individuals who were vaccinated using a separate batch. Lurmen concluded that vaccination was the "etiological source of the icterus epidemic" (Lürman 1885). Later, Stokes reported that the incidence of jaundice was 10 times higher in patients who received intravenous arsphenamin for syphilis at the Mayo Clinic, USA, from 1917 to 1920. After excluding arsphenamine and syphilis, he suggested infection spread by hematogenous means as the possible etiology (Stokes et al. 1920). Similar observations and conclusions were drawn by McCallum in 1943 in venereal disease centres across the USA (MacCallum 1943).

9.3.5 World War II: Key Observations About Changing Patterns of Hepatitis

In the meantime, the association between jaundice and wars was once again witnessed. In World War II, around 2 lakh cases of jaundice were reported in the US troops. More than 50 lakh cases were reported amongst the Germans including the military and civilian population (Wong et al. 2015). Several outbreaks were reported in the Mediterranean coast, North Africa and West Asia. On the flip side, this had a hidden benefit. The Allied powers, particularly the USA and Great Britain, invested significant amounts of money and infrastructure in research in hepatitis in order to gain a strategic advantage over the Germans.

During World War II, a new epidemiological pattern began to emerge. Many of the cases were related to vaccination, sharing of syringes and needles, and transfusion of blood or plasma. The US War Secretary declared that between 1st January and 4th July, 1942, approximately 28,000 cases of jaundice developed following vaccination against yellow fever with a mortality of 0.22% (Dooley 2005). The Health Ministry of Great Britain published a document titled "Homologous Serum Jaundice" in 1943, which reported on outbreaks of jaundice following transfusion of plasma and blood. They reviewed the available literature and made the very important observation that the incidence of hepatitis in institutions that used washed syringes and shared needles ranged from 30 to 60% compared to practically zero in institutions that used sterilized or boiled syringes. They correctly surmised that the hepatitis agent was transmitted during "venipuncture and intravenous injections" (MacCallum 1947).

9.3.6 Existence of Two Types of Hepatitis: A Vital Piece of the Puzzle

These observations led to the recognition that there were two distinct types of hepatitis based on clinical and epidemiologic characteristics. The first type ("infectious hepatitis") was characterised by a brief incubation period, faeco-oral transmission, and was often clustered in outbreaks and epidemics. The second type ("serum hepatitis") had a longer incubation period and was transmitted by injections or transfusion of blood and blood products. Further, patients with prior infectious hepatitis were immune to new onset "infectious hepatitis" while patients with "serum hepatitis" had no immunity against "infectious hepatitis". The conclusions drawn were remarkably accurate. Indeed, as early as 1947, McCallum suggested the nomenclature of Hepatitis A and B, well before the discovery of the respective viruses (Tandon et al. 1996; MacCallum 1947).

9.3.7 The Dark Phase of Human Experimentation

The history of the discovery of hepatitis viruses would not be complete without alluding to the questionable ethics of some of the studies that were carried out during this time, particularly during World War II and the immediate post-war period. One of the vexing problems facing researchers at this time was that humans appeared to be the only hosts for the infective agents of hepatitis and all attempts to grow the agent in cellular cultures and animals were futile. We have alluded to the remarkable conclusions about the distinct forms of hepatitis that were made in the 1940s. On a darker note, many of these conclusions were drawn from human experimentation. Various researchers, including McCallum, Stokes, Havens and Neefe, carried out research that involved inoculating healthy human volunteers with faeces, serum or duodenal fluids from patients with hepatitis (Tandon et al. 1996; Khuroo and Sofi 2020; Trepo 2014). Human experimentation in hepatitis continued through the next two decades. A wealth of information on the epidemiology and natural history of hepatitis was obtained from a series of human experiments that were carried out on mentally challenged children at Willowbrook State School, a government-funded hostel cum in New York from 1956 to 1971 (Krugman 1986). Krugman was both the protagonist and villain who was credited and later blamed for these trials. While the studies had no methodological flaws and there is no reason to doubt the honest and scientific intentions of Krugman, they generated a substantial amount of debate on ethics in medical research particularly with respect to informed consent and mental competency to give the same (Goldby 1971). However, many leading scientists of that time had spoken out in favour of Krugman.

While these appalling practices are indefensible, it should be noted that human sensibilities and ethics evolve with time. With advancements in tissue engineering and the development of organoids, it is not difficult to foresee that several decades into the future, new drugs would be tested on fully functional 3-dimensional tissue-organ systems instead of human volunteers. The current practice of controlled

human trials may well be deemed as unethical by the future generations of researchers.

9.4 Serendipitous Discovery of Hepatitis B

Baruch Blumberg was a geneticist who was primarily interested in studying if differences in serum lipoproteins were linked to the genetic susceptibility to disease. While working at the National Institute of Health (NIH), Bethesda, USA, he studied blood samples obtained from indigenous populations in remote corners of the world to see if serum protein polymorphisms were related to disease susceptibility. He also collected blood samples of haemophilia patients who had received multiple blood transfusions. He believed that these patients who had received transfusions from unrelated donors would have antibodies against the pleomorphic serum proteins that could be detected by immunodiffusion in agar-gel. In 1965, his team published the seminal paper on the discovery of the Australia antigen in an Australian aborigine which showed immunologic reaction with the sera of the patients with haemophilia (Krugman 1986). The Australia antigen is now recognised to be the surface antigen of the Hepatitis B virus (HBsAg). Blumberg looked for antibodies against this new antigen in the sera of 107 patients with a history of multiple blood transfusions and detected it in 10.3% patients. They then tested the sera of 1704 healthy individuals and 659 patients to determine the prevalence of antibodies against the "Australia antigen". While none were detected among Americans, antibodies were present in 3.8% of foreigners and 11.4% patients with leukaemia (Krugman 1986). Blumberg speculated that the Australia antigen may increase the susceptibility of leukaemia or may be related to a leukaemia-causing virus and even suggested that it may be useful in the early diagnosis of leukaemia. He could not have been further away from the truth.

It was the team of Alfred Prince at the New York Blood Centre who unravelled the significance of Blumberg's mysterious discovery. He identified a novel antigen (designated as SH) in the serum of a haemophiliac with multiple transfusions who had developed "serum hepatitis" following a surgery for bleeding peptic ulcer which had required multiple transfusions (Goldby 1971; Blumberg et al. 1965). The patient's serum had been collected in the intervening incubation period prior to the development of hepatitis. Prince subsequently demonstrated that the SH antigen was present in almost 80% patients with serum hepatitis and only 0.1% of healthy individuals. Further, it was found that the Australia antigen and SH antigen were similar and while this antigen was associated with "serum hepatitis", there was no association with "infectious hepatitis" (Prince 1968).

The serendipitous nature of Blumberg's discovery can be easily understood from the words of Harvey Alter, who was the second author in the landmark paper on Australia antigen. He wrote, "the chronological events surrounding the Australia antigen stand out as a monument to non-directed medical research and as a tribute to investigative perseverance. This tale of serendipity began in the mid-1960s when the Australia antigen was first reported by a geneticist who had been seeking new

inherited polymorphisms among serum proteins, by a blood banker looking for non-cellular causes of febrile, non-haemolytic transfusion reactions and by a technologist destined to become a commercial airline pilot. A research interest in viral hepatitis was conspicuously absent in this investigative team. The significance of the Australia antigen, found when the serum of an Australian aborigine formed a precipitin line with the serum of a multiply transfused haemophiliac, was, at that time, unknown" (Alter 1981).

In 1976, Blumberg was honoured with the Nobel Prize in Medicine for the discovery of the Hepatitis B virus. Many authorities feel that the exclusion of Prince from this prestigious award was a travesty. Nonetheless, the contributions of Blumberg in Hepatitis B research were humongous.

He continued his work at the Institute of Cancer Research in Philadelphia and found that the Australia antigen was present in the sera of 20% patients presenting with acute hepatitis. He also showed a high prevalence of the Australia antigen in patients with Downs syndrome who had abnormal liver function tests on blood biochemistry with evidence of hepatitis on liver biopsy, and in patients with post-transfusion hepatitis. He thus surmised that this antigen was probably derived from a virus that caused hepatitis (London et al. 1969). He also noted that the Australia antigen had a diameter of 20 μm (London et al. 1969). The complete 42 μm viral particle was identified by Dane et al. in 1970 (Dane et al. 1970). By treating the Dane particles with detergent, Almeida et al. subsequently demonstrated that the "Australia antigen" was the surface antigen of the Hepatitis B virus (HBs Ag) and also found another antigen known as the core antigen (Almeida et al. 1971). HBs Ag was found to be non-infective but immunogenic and an excellent surrogate marker for the presence of Hepatitis B infection. Blumberg along with Millman also received the first patent for a prototype Hepatitis B vaccine in 1969 using HBs Ag isolated from the blood of infected patients (Blumberg 2002). Using the principles demonstrated by Blumberg, Maurice Hilleman, a researcher working at Merck developed Heptavax B, which was the first Hepatitis B virus vaccine to be approved by the FDA in 1983 (Szmuness et al. 1981).

9.5 Discovery of Hepatitis C: A Tale of Innovation and Collaboration

9.5.1 Non-A Non-B Hepatitis

The possibility of a second parenterally transmitted hepatitis virus was suggested by Gocke et al. in 1970 when they reported that many of the cases of post-transfusion hepatitis occur in patients who have been received blood from donors who were negative for Australia antigen (Gocke et al. 1970). At that time, a definitive conclusion could not be drawn due to constraints in the accuracy of the agar gel diffusion technique described by Blumberg. A sentinel moment in research in viral hepatitis was the development of a radioimmunoassay technique for detecting even small amounts of HBs Ag (Australia antigen) and its antibody. This test was easier to

perform and was more accurate than the agar gel diffusion technique (Walsh et al. 1970).

Using this improved armamentarium of reliable serological tests for detecting both Hepatitis B and Hepatitis A, Harvey J. Alter and his colleagues working at the Department of Blood Transfusion in the Bethesda centre of NIH, USA, found that a considerable number of cases of post-transfusion hepatitis was attributable to neither Hepatitis A nor Hepatitis B. This entity was popularly referred to as non-A non-B hepatitis. Using injectable extracts prepared from the serum of patients with non-A non-B hepatitis, Alter successfully transmitted the disease in five chimpanzees in 1974. He demonstrated that all chimpanzees developed deranged liver function parameters and features of hepatitis on liver biopsy after an average incubation period of 13.4 weeks (Alter et al. 1972, 1978; Feinstone et al. 1975). His findings were confirmed by Tabor et al. who transmitted non-A non-B hepatitis to four chimpanzees who subsequently developed hepatitis after a prolonged incubation period (Tabor et al. 1978). The scientific community quickly inferred that the transmissibility of non-A non-B hepatitis and other findings implicated an infective agent probably a virus.

9.5.2 Search for the Virus: A Decade of Failures

The search began in earnest to identify this novel entity. However, all efforts to detect the virus over the next 15 years were in vain. The virus eluded detection by all the traditional methods of microscopy, culture and serology. At least 19 claims to have found the virus were made during this time but all of them failed to show immunological reactivity with a well characterised serum pool prepared by Alter from patients with non-A non-B hepatitis (Tandon et al. 1996).

9.5.3 A Game-Changing Innovation Backed Up by Dogged Perseverance

In the early 1980s, the application of molecular techniques was in a fledgling state. The implementation of this approach by Michael Houghton and his team at Chiron Corp., California, USA ultimately led to the identification of the virus. Houghton and his colleagues, Qui-Lim Choo and George Kuo, collaborated with Daniel Bradley of the Centre of Disease Control and Prevention (CDC), Atlanta, USA and developed a library of cDNA using hepatic and pancreatic tissue samples obtained from chimpanzees infected with non-A non-B hepatitis. These were then transferred to bacteria using lambda bacteriophages and cloned. Serum of patients with non-A non-B hepatitis was used as a likely source of antibodies. The hypothesis was that some of the proteins encoded by these cDNA would immunologically react with these antibodies in the serum of patients with non-A non-B hepatitis.

In 1989, after 6 years of painstaking research, the first such antigen epitope (corresponding to the viral envelope) was identified from a clone called 5-1-1.

This clone hybridised with a single-stranded RNA fragment of 10,000 nucleotides obtained from infected chimpanzees but no such phenomenon was observed with genetic material obtained from non-infected chimpanzees. This finding suggested that the genetic material of the clone was present only in infected chimpanzees and possibly originated from a RNA virus. Further, they demonstrated that the antigen expressed by the clone 5-1-1 showed immunological reaction with antibodies present in the serum of chimpanzees infected with non-A non-B hepatitis and also with the isolate that had been prepared and characterised by Alter (Choo et al. 1989). The expressed antigen did not cross-react with antibodies against Hepatitis A and Hepatitis B. Moreover, patients with non-A non-B hepatitis were found to be seropositive for antibodies against 5-1-1 (Kuo et al. 1989). The etiologic agent of non-A non-B hepatitis was finally thus identified. This positive-stranded RNA virus was named Hepatitis C and was subsequently classified as a Flavivirus.

The novel methods adopted by Houghton drastically changed the field of virology and heralded a molecular revolution. Innovations and refinements in molecular techniques like PCR allowed the sequencing of the whole genome of the SARS COVID-19 within weeks of it first being reported in China. It is also prudent to reflect on the numerous failures in the period between Alter's work in chimpanzees in 1974 and Houghton's discoveries in 1989. Houghton's team themselves went through 6 years of exasperating failures. Perseverance is key in research. Failure in research and indeed any aspect of life is not a cause for concern but rather a lesson in "what and how not to do".

9.5.4 The Final Piece of the Puzzle

By the beginning of the 1990s decade, scientists had discovered the modes of transmission of HCV, established its infectivity in chimpanzees and unravelled its nuclear structure. However, whether infection with the virus alone was sufficient to cause clinically significant disease was yet unknown. Charles M. Rice and his team at Washington University, USA and Kunitada Shimotohno who was leading a Japanese research team characterised a highly conserved region at the 3′ non-translated end of the Hepatitis C viral genome (Kolykhalov et al. 1996; Tanaka et al. 1996). Rice correctly surmised that the highly conserved nature suggested that it was important in viral replication. Rice and Alexander Kolykhalovthen constructed a library of cDNA clones using an isolate previously characterised by Alter. A consensus sequence was developed by Sanger's sequencing of many cDNA sequences followed by reconstruction using restriction enzymes. A complete genomic clone was ultimately genetically engineered using the previously described 3′ untranslated region of the Hepatitis c viral genome and the consensus sequence. RNA transcribed from these cDNA clones was infective to chimpanzees and resulted in hepatitis (Kolykhalov et al. 1997). Thus, the link between Hepatitis C virus and liver disease was firmly established.

9.6 The Landscape of Hepatitis B and C in 2020: Reaping the Benefits

Bar-Gal et al. recently isolated the complete genome of Hepatitis B from hepatic extracts of a Korean mummy (Bar-Gal et al. 2012). Dating estimates of the most recent common ancestor places the origins of this ancient Hepatitis B sequence to be 3000–100,000 years old. Yet, within six decades of the discovery of the viruses implicated in chronic hepatitis, we have developed ways to diagnose, prevent and treat them. Indeed, the pace of advancement of our knowledge and technical prowess is exponential once the initial spark has been lit by stalwarts.

Highly accurate, readily available and cheap serological tests have facilitated the easy screening of Hepatitis B and C infections. Routine screening of all blood donors for antibodies against these viruses is now the norm all over the world. Recognition of the modes of viral transmission led to the adoption of universal safety precautions which has greatly reduced occupational risk in health care workers. Hepatitis B vaccination is now a part of the universal immunisation schedule. Importantly, as Hepatitis B is a leading cause of liver cancer, this vaccine is also the first anti-cancer vaccine.

The non-specific antiviral agent interferon (discovered in 1957) was the first drug that was approved for the treatment of Hepatitis B in 1991 (Trepo 2014). In the same year, lamivudine, a nucleoside analogue, was shown to prevent the replication of Hepatitis B by inhibiting RNA-dependent DNA polymerase (an enzyme involved in reverse transcription). In 1998, Lamivudine was the first of several oral drugs belonging to a class known as "nucleoside inhibitors" that was approved for the treatment of Hepatitis B (Trepo 2014). Although highly effective, lamivudine and many other "nucleoside inhibitors" are constrained by the development of resistance due to genetic mutations. Currently, "nucleoside inhibitors" with a high barrier to resistance like tenofovir are used for treating Hepatitis B. Pegylated Interferon α also continues to be a viable treatment option although it is uncommonly used in India and most parts of the world because of adverse effects, tolerability issues and need for injections. The main problem with currently available Hepatitis B therapies is that while they are excellent in supressing the viral load, they cannot eliminate the infection completely. This is because some of the Hepatitis B DNA exists as covalently closed circular DNA (cccDNA) in a plasmid-like form inside the host nucleus and acts as a template for progeny production. cccDNA cannot be effectively targeted by currently available means. Elimination of cccDNA is the holy grail of research in Hepatitis B management. Epigenetic modulation and gene editing techniques like the CRISPR-CAS system hold potential in targeting cccDNA with the promise of "complete cure" in the future (Lok et al. 2017).

Long an unmet meet, the treatment of Hepatitis C was revolutionised in the past decade. Treatment failures and adverse effects were frequent in the interferon era. Research into directed therapies against Hepatitis C was greatly hindered in the 1990s by the absence of a method to maintain and propagate the virus in-vitro. The development of replicons by Bartenschlager and Lohmann in 1999, was the next momentous breakthrough in Hepatitis C research (Lohmann et al. 1999). These

subgenomicrepicons were bicistronic RNA constructs that could replicate autonomously in human hepatoma-derived Huh-7 cells. Replicons facilitated the granular understanding of the Hepatitis C genome and its structural and non-structural proteins, precise characterisation of the viral replication complex, and permitted the screening and testing of directly acting antivirals (DAAs) in vitro. In the current generation, oral DAAs have efficacy rates of >95–99% with minimal adverse effects (Baumert et al. 2019). They represent a paradigm shift in the management of Hepatitis C and are offering the hope of cure to millions of patients. An effective vaccine against Hepatitis C is still lacking. Despite the availability of highly effective treatment, a vaccine is still deemed necessary to attain the ultimate goal of elimination of the scourge of Hepatitis C.

9.7 Conclusion

The story of the unravelling of the mysteries behind "serum hepatitis" is an incredible testament to the remarkable accomplishments of modern biomedical research. Acknowledgement of the work of these "masters of science" and the remarkably long way that we have come in our understanding of Hepatitis B and C provides much-needed encouragement to researchers as they try to fight the hitherto unknown threat of COVID-19.

Conflict of Interest and Financial Disclosures All authors have no financial disclosures and declare no conflict of interest.

References

Almeida JD, Rubenstein D, Stott EJ (1971) New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet 2:1225–1226

Alter HJ (1981) Hepatitis B: a tribute to nondirected medical research. Semin Liver Dis 1:1–6

Alter HJ, Holland PV, Purcell RH, Lander JJ, Feinstone SM, Morrow AG et al (1972) Posttransfusion hepatitis after exclusion of commercial and hepatitis-B antigen-positive donors. Ann Intern Med 77:691–699

Alter HJ, Purcell RH, Holland PV, Popper H (1978) Transmissible agent in non-A, non-B hepatitis. Lancet 1:459–463

Bar-Gal GK, Kim MJ, Klein A, Shin DH, Oh CS, Kim JW et al (2012) Tracing hepatitis B virus to the 16th century in a Korean mummy. Hepatology 56:1671–1680

Baumert TF, Berg T, Lim JK, Nelson DR (2019) Status of direct-acting antiviral therapy for hepatitis C virus infection and remaining challenges. Gastroenterology 156:431–445

Blumberg BS (2002) Hepatitis B: the hunt for a killer virus. Princeton University Press, Princeton and Oxford, pp 72–146

Blumberg BS, Alter HJ, Visnich S (1965) A "new" antigen in leukemia sera. JAMA 191:541–546

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362

Cooke GS, Andrieux-Meyer I, Applegate TL, Atun R, Burry JR, Cheinquer H et al (2019) Accelerating the elimination of viral hepatitis: a Lancet Gastroenterology & Hepatology Commission. Lancet Gastroenterol Hepatol 4:135–184

Dane DS, Cameron CH, Briggs M (1970) Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet 1:695–698
Dooley DP (2005) History of U.S. military contributions to the study of viral hepatitis. Mil Med 170:71–76
Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV (1975) Transfusion associated hepatitis not due to viral hepatitis type A or B. N Engl J Med 292:767–770
Gocke DJ, Greenberg HB, Kavey NB (1970) Correlation of Australia antigen with post-transfusion hepatitis. JAMA 212:877–879
Goel A, Seguy N, Aggarwal R (2019) Burden of hepatitis C virus infection in India: a systematic review and meta-analysis. J Gastroenterol Hepatol (Australia) 34:321–329. https://doi.org/10.1111/jgh.14466
Goldby S (1971) Experiments at the Willowbrook State School. Lancet 1:749
Gruber W, Virchow R (1865) Ueber das Vorkommen und den Nachweis des hepatogenen, insbesondere des katarrhalischen Icterus. Arch Patholog Anat Und Physiol Und Für Klin Med 32:117–125
Khuroo MS, Sofi AA (2020) The discovery of hepatitis viruses: agents and disease. J Clin Exp Hepatol 10:391–401. https://doi.org/10.1016/j.jceh.2020.04.006
Kolykhalov AA, Feinstone SM, Rice CM (1996) Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. J Virol 70:3363–3371
Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 277:570–574
Krugman S (1986) The Willowbrook hepatitis studies revisited: ethical aspects. Rev Infect Dis 8:157–162
Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH et al (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364
Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113
Lok AS, Zoulim F, Dusheiko G, Ghany MG (2017) Hepatitis B cure: from discovery to regulatory approval. J Hepatol 67(4):847–861
London WT, Sutnick AI, Blumberg B (1969) Australia antigen and acute viral hepatitis. Ann Intern Med 70:55–59
Lürman A (1885) Eine Icterus Epidemic. Berlin Klin Wochenschr 22:207
MacCallum FO (1943) Jaundice in syphilitics. Br J Vener Dis 19:63
MacCallum FO (1947) Homologous serum jaundice. Lancet 2:691–692
Murchison C, ByIhird LB, Fayrer J (1886) Clinical lectures on diseases of the liver, jaundice, and abdominal dropsy. Biomed J Digit Project 31
Prince AM (1968) An antigen detected in the blood during the incubation period of serum hepatitis. Proc Natl Acad Sci U S A 60:814–821
Schmidt PJ (1999) Blood: an epic history of medicine and commerce. Transfusion 39:793–793. https://doi.org/10.1046/j.1537-2995.1999.39070793.x
Sood A, Suryaprasad A, Trickey A, Kanchi S, Midha V, Foster MA et al (2018) The burden of hepatitis C virus infection in Punjab, India: a population-based serosurvey. PLoS One 13:1–18. https://doi.org/10.1371/journal.pone.0200461
Stokes JH, Ruedemann R Jr, Lemon WS (1920) Epidemic infectious jaundice and its relation to the therapy of syphilis. Arch Int Med 26:521–543
Szmuness W, Stevens CE, Zang EA, Harley EJ, Kellner A (1981) A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report. Hepatology 1(5):377–384
Tabor E, Gerety RJ, Drucker JA et al (1978) Transmission of non-A, non-B hepatitis from man to chimpanzee. Lancet 1:463–466
Tanaka T, Kato N, Cho MJ, Sugiyama K, Shimotohno K (1996) Structure of the 3′ terminus of the hepatitis C virus genome. J Virol 70:3307–3312

Tandon BN, Acharya SK, Tandon A (1996) Epidemiology of hepatitis B virus infection in India. Gut 38(Suppl 2):S56–S59

Trepo C (2014) A brief history of hepatitis milestones. Liver Int 34:29–37. https://doi.org/10.1111/liv.12409

Walsh JH, Yalow RS, Berson SA (1970) Radioimmunoassay of Australia antigen. Vox Sang 19(3): 217–224

WHO (2017) Global hepatitis report. World Health Organization, Geneva. http://apps.who.int/iris/bitstream/10665/255016/1/9789241565455-eng.pdf

Wong DT, Martin CM, Boyer JL, Jain D (2015) Historical path of discovery of viral hepatitis. HMSR 3:18–36

Conventional and Modern Approaches for Clinical and Laboratory Diagnosis of Tuberculosis

10

Meenu Kaushal Sharma, Quinn Wonitowy, and Hafid Soualhine

Abstract

The *Mycobacterium tuberculosis* complex (MTBC) is comprised of species *M. tuberculosis, M. bovis* including *M. bovis BCG* (vaccine strain)*, M. caprae, M. pinnipedii, M. africanum, M. microti, M. canettii, "M. mungi",* and *"M. orygis"* (Woods et al., Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes: approved standard, Wayne, 2011). Mycobacteria are acid-fast bacilli and unlike most other bacteria, they have lipid-rich cell walls and due to the presence of mycolic acid, their cell walls are impermeable to a variety of disinfecting and antimicrobial agents. This makes them resistant to a variety of chemical and pharmaceutical agents. MTBC can survive harsh climates, varying temperatures and can live in deceased hosts for long periods of time (e.g. mummies). Chronic granulomatous disease caused by *M. tuberculosis* has manifestations, involving primarily lungs but sometimes other organ systems as well. MTBC are 1–10 μm in length, aerobic, non-motile, and slowly growing bacteria with 18–20-h doubling time. MTBC smear morphology shows rods that are known for their serpentine cording due to cord factor trehalose 6, 6 dimycolate. Clinical diagnosis can be done by chest X-ray, mantoux test or symptom check in conjunction with risk factors. Laboratory testing includes smear microscopy, interferon-gamma release assays, culture, rapid-detection, identification, antimicrobial susceptibility testing, and genotyping.

M. K. Sharma (✉) · H. Soualhine
National Reference Centre for Mycobacteriology, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada

University of Manitoba, Winnipeg, MB, Canada
e-mail: meenu.sharma@phac-aspc.gc.ca

Q. Wonitowy
University of Manitoba, Winnipeg, MB, Canada

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_10

Whole-genome sequencing is currently becoming a new norm for direct direction, identification, antimicrobial susceptibility prediction or confirmation and outbreak/contact tracing/contamination investigations. Whole-genome sequencing results in large amounts of data and the bioinformatic tools for analyzing this data remain complex.

Keywords

Mycobacterium tuberculosis · Isoniazid · Rifampin · Antimicrobial susceptibility testing · Genotyping · Whole genome sequencing

10.1 Introduction

10.1.1 *Mycobacterium tuberculosis* and Tuberculosis

Mycobacterium tuberculosis is a pathogenic bacterium belonging to phylum actinobacteria, order actinomycetales and mycobacteriacae family. *M. tuberculosis* primarily infects the lungs and is the causative agent for tuberculosis (TB) infection but can manifest in many organ systems such as cerebrospinal fluid, etc. The bacterium can linger around in infected organs for years and decades. Infection occurs when bacteria are released from a contagious individual via coughing or other expulsions with excessive force. The infectious dose is 1–10 *M. tuberculosis* bacilli and a droplet particle generated by an infectious person can contain 1–3 or more bacterial cells (World Health Organization 2020; Forbes et al. 2018). These droplets can be inhaled by another individual and infect pulmonary macrophages of the host.

Active infection is a symptomatic form of TB disease that is culture positive for *M. tuberculosis*. A person with this type of disease may be infectious and require isolation. Active infection can develop in approximately 5–10% of infected individuals. The first infection is termed as primary TB (World Health Organization 2020; Forbes et al. 2018), which may go unnoticed in 90–95% of individuals, become latent, and remain so for a person's lifetime. Primary TB presents with hilar enlargement, unilateral parenchymal infiltrates and/or pleural fluid. The linear or alveolar densities are usually small and appear early as small calcified 'granulomatous' lesions predominantly in the lower lobes. In active TB, Ghon complex may be a pathological indicator of TB disease progression. The lesion in lungs is of a granulomatous inflammation and adjacent lymph node. Miliary TB represents unchecked haematogenous dissemination of mycobacteria resulting in foci either at the time of primary disease or later during reactivation. Reactivation of TB typically presents with infiltrates in the upper lung zones with or without cavitations or with a miliary pattern TB. Extrapulmonary TB is a disease of other human organs (not lungs) such as TB lymphadenitis, pleural TB, genitourinary TB, skeletal TB, meningeal TB or pericardial TB (Centers for Disease Control and Prevention 2016a, d; World Health Organization 2020; Forbes et al. 2018).

Latent TB infection or LTBI is an asymptomatic form of TB infection that is smear- and culture-negative for *M. tuberculosis*. In these individuals, infection remains under immune control, which is effective at limiting infection (Centers for Disease Control and Prevention 2016a, b, c). A healthy individual can harbour a LTBI for their lifetime, but in instances where the immune system becomes weakened such as immunosupression, HIV, autoimmune disease etc., the dormant bacteria can become active in 5–10% of the cases and is termed as secondary TB (World Health Organization 2020; Centers for Disease Control and Prevention 2016a, b, c).

10.1.2 Tuberculosis Epidemiology

TB is the leading cause of death, globally, from a single infectious bacterial agent. In 2019, TB caused disease in 10 million people worldwide, resulting in 1.2 million deaths from TB among HIV-negative individuals and 208,000 deaths in HIV-positive individuals (World Health Organization 2020). SARS CoV-2 infections have hindered TB diagnosis and treatment globally due to limited medical access. The majority of TB cases globally occur in Africa and Southeast Asia and the Western Pacific regions, with global percentages of 25%, 44%, and 18% respectively (Table 10.1, Fig. 10.1). In contrast, Europe and the Americas harbour only 2.5% and 2.9% of global TB cases (World Health Organization 2020). Eight countries account for 2/3rd of the global TB burden namely in descending order: India, Indonesia, China, Philippines, Pakistan, Nigeria, Bangladesh and South Africa (Table 10.1). From 2015 to 2019, a total of 78 countries are on track to reach the 2020 milestone of a 20% reduction in TB incidence. In 2019, an estimated 3.3% of new TB cases and 18% of previously treated cases had multi-drug-resistant TB (i.e. there were an estimated 465,000 incident cases of rifampicin-resistant TB); 78% had multi-drug-resistant TB. India (27%), China (14%) and the Russian Federation (8%) had the largest share of the global burden (World Health Organization 2018). Overall, drug resistance of *M. tuberculosis* in Canada occurs at relatively low rates (LaFreniere et al. 2018). 8.1% of tested isolates in 2017 were resistant to at least one first-line anti-TB drug; most were mono-resistant and no tested isolates were XDR-TB (LaFreniere et al. 2018).

10.2 Drug Resistance in Tuberculosis

The inappropriate and inadequate antibiotic use for the treatment of bacterial infections results in the development of drug-resistant bacteria, which has progressively made the treatment of infections more cumbersome (Laxminarayan et al. 2013). *M. tuberculosis,* in particular, antibiotic-resistant organisms are more difficult to treat, can increase the cost of treatment, time to recovery, and rate of patient mortality (World Health Organization 2020). While many bacterial species are able to gain resistance genes through horizontal gene transfer (HGT), *M. tuberculosis*

Table 10.1 Best estimated values for epidemiological burden of TB in 2019 for 30 high burden countries, WHO region and globally (rates per 100,000 population). © World Health Organization (2020). Global tuberculosis report 2020. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo)

Country	Total TB incidence[a]	HIV prevalence among incident TB cases (%)	HIV-negative TB mortality	HIV-positive TB mortality[b]
Angola	351	7.6	53	8.2
Bangladesh	221	0.19	24	0.093
Brazil	46	11	2.3	0.87
Cambodia	287	2.7	17	2.5
Central African Rep.	540	25	98	61
China	58	1.6	2.2	0.15
Congo	373	29	52	40
DPR Korea[c]	513	–	–	–
DR Congo	320	11	49	11
Ethiopia	140	6.5	19	2.5
India[d]	193	2.7	32	0.69
Indonesia	312	2.2	34	1.7
Kenya	267	26	37	24
Lesotho	654	62	57	168
Liberia	308	14	56	17
Mozambique	361	34	19	18
Myanmar	322	7.8	36	5.8
Namibia	486	32	57	50
Nigeria	219	11	63	14
Pakistan	263	0.90	19	0.90
Papua New Guinea	432	3.8	47	3.5
Philippines	554	1.9	25	0.75
Russian Federation	50	23	5.8	0.88
Sierra Leone	295	13	31	8.7
South Africa	615	58	38	62
Thailand	150	10	14	2.8
UR Tanzania	237	24	35	20
Vietnam	176	3.3	9.8	2.0
Zambia	333	46	33	53
Zimbabwe	199	60	11	31
High TB burden countries	**177**	**7.8**	**21**	**3.4**
Africa	226	24	35	16
The Americas	29	10	1.7	0.58
Eastern Mediterranean	114	0.97	11	0.38

(continued)

Table 10.1 (continued)

Country	Total TB incidence[a]	HIV prevalence among incident TB cases (%)	HIV-negative TB mortality	HIV-positive TB mortality[b]
Europe	26	12	2.2	0.45
South-East Asia	217	2.7	32	1.0
Western Pacific	93	2.0	4.4	0.33
Global	**130**	**8.2**	**16**	**2.7**

[a]Rate per 100,000; numbers rounded off to significant figures
[b]Deaths among HIV-positive TB cases are classified as HIV deaths
[c]TB incidence for DPR Korea not yet approved by national authorities
[d]Estimates for India are interim, pending results from the national TB prevalence survey (2020/2021)

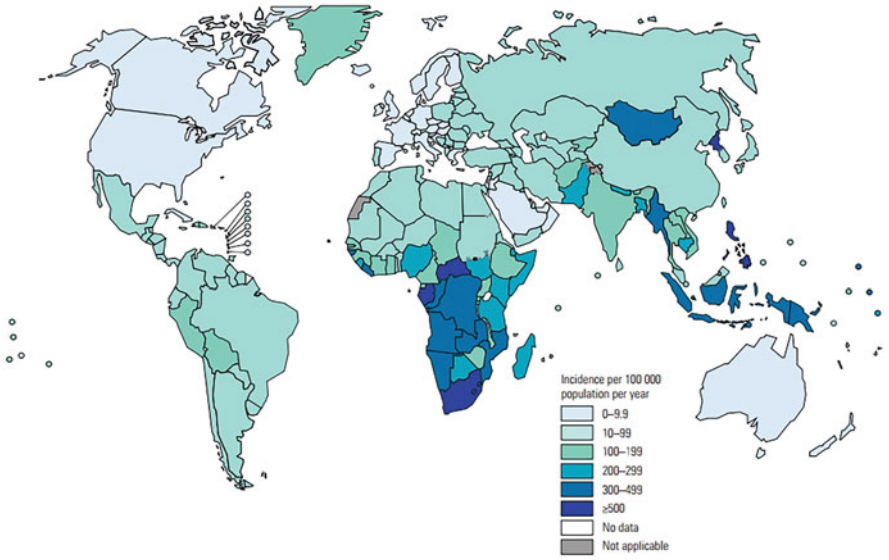


Fig. 10.1 Estimated TB incidence rates, 2019. © World Health Organization (2020). Global tuberculosis report. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO

strains gain spontaneous genomic mutations leading to resistance (Davies and Davies 2010; Schürch and Schaik 2017; Eldholm and Balloux 2016).

10.2.1 Mode of Action of Antimicrobials and Mechanisms of Drug Resistance in *M. tuberculosis*

Eldholm et al. (2014) found that *M. tuberculosis* isolates, further to evolving mutations leading to antibiotic resistance, also can independently increase fitness

over time in the presence of antibiotics. The existence of a heterogeneous population infecting an individual can further complicate treatment, as variable drug susceptibilities may be present (Nathavitharana et al. 2017). Distinct lineages of *M. tuberculosis* exist, and different mutation rates in each lineage subsequently has led to different resistances in each lineage (Ford et al. 2013). For example, strains of Lineage 2 are more likely to be resistant to multiple drugs (Ford et al. 2013). Much of the *M. tuberculosis* genomes undergo purifying selection, though antibiotics can provide pressure for positive selection of resistance mutations (Pepperell et al. 2013; Mortimer et al. 2018).

TB diagnosis and treatment is complicated due to slow growth rate of the bacteria, it's rigid and impermeable cell wall, and length and side effects of the prescribed medications. The mycolic acid content in the cell wall primarily contributes to decreased permeability of some antibiotics (Nikaido 1994). *M. tuberculosis* also has the ability to combat anti-tuberculosis drugs with various efflux systems (Balganesh et al. 2012). *M. tuberculosis* resistance is a growing problem, and has led to treatment courses that are of longer duration, more expensive and more difficult to maintain (Public Health Agency of Canada 2014). In 2016, Gallant et al. (2017) found that 9% of tested isolates were resistant to at least one drug. 83.1% of these exhibited monoresistance, this being more common than multi- or extensive-resistance.

10.2.1.1 Isoniazid

Isoniazid is a synthetic derivative of nicotinic acid with anti-mycobacterial properties. Isoniazid or INH is converted into its active form by the catalase-peroxidase enzyme encoded by the *katG* gene (Fig. 10.2) (Zhang et al. 1992). INH forms an adduct with NAD(H); competing with NAD(H) in binding to the *inhA* gene product, enoyl-acyl carrier protein reductase (Zhang et al. 1992). The gene product of *inhA* is involved in mycolic acid biosynthesis in the mycobacterial cell wall. The activated form of INH interferes with mycolic acid synthesis, making the cell wall

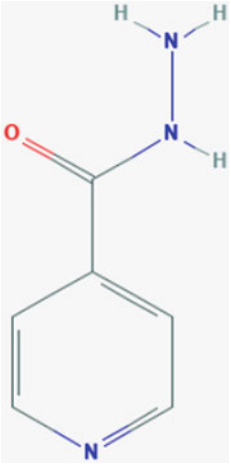


Fig. 10.2 Molecular structure of Isoniazid ($C_6H_7N_3O$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 3767, Isoniazid" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Isoniazid. Accessed 3 June 2021)

fragile (Banerjee et al. 1994). The two main causes of INH resistance include: mutations in the *katG* gene; and mutations in the *inhA* gene and promoter (Banerjee et al. 1994). Mutations in the *katG* gene lead to a decrease or to the loss of enzyme activity. Point mutations, insertions, deletions and truncations have all been identified in *katG*-mediated INH resistance (Zhang et al. 1992).

Mutations in the *inhA* promoter cause the overexpression of enoyl-acyl carrier protein reductase. Mutations in the promoter region are frequently reported in mono-resistant strains. Resistance to this drug has been associated with mutations in several genes, such as *katG, inhA, ahpC, kasA* and NDH (Palomino and Martin 2014). There is also evidence to suggest that efflux pumps and ABC transporters play a role in INH resistance (Colangeli et al. 2005; Jiang et al. 2008).

10.2.1.2 Rifampin

Rifampin or Rifampicin is a member of the class of rifamycins that is a semisynthetic antibiotic derived from *Amycolatopsis rifamycinica* (Fig. 10.3). Table 10.2 shows global incidence of rifampin resistant TB. Rifampin or RMP targets the β-subunit of RNA polymerase. The drug binds the β-subunit of the enzyme, physically blocking RNA polymerase, and thereby inhibiting RNA transcription (Blanchard 1996; Somoskovi et al. 2001, Forbes CLSI). Resistance to rifampin has been linked to mutations within the *rpoB* gene that encode the β-subunit of RNA polymerase. Within *rpoB* is an 81 bp rifampin resistance-determining region (RRDR) which is

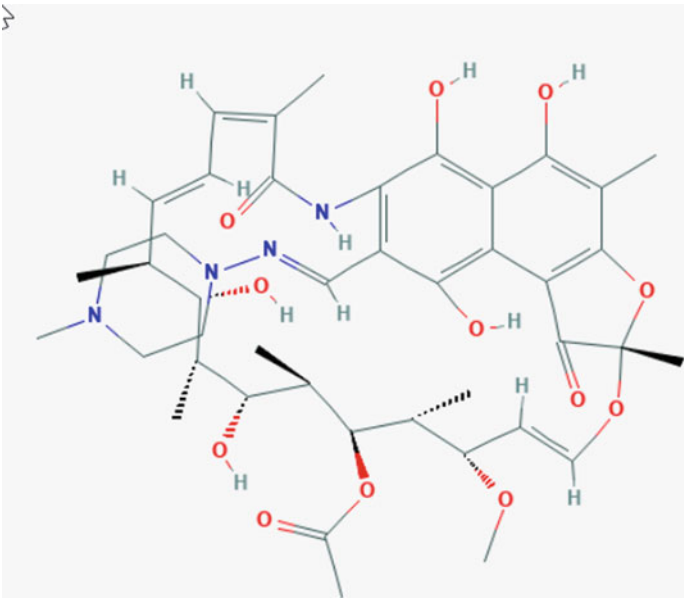


Fig. 10.3 Molecular structure of Rifampin ($C_{43}H_{58}N_4O_{12}$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 135398735, Rifampicin" PubChem, https://pubchem.ncbi.nlm.nih.gov/compound/Rifampicin. Accessed 3 June 2021)

Table 10.2 Estimated global incidence of rifampicin-resistant and/or isoniazid-resistant TB, 2019 (Number in thousands) © World Health Organization (2020). Global tuberculosis report 2020. Geneva: World Health Organization (2020). Licence: CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo)

	Rifampin resistant Best estimate	Rifampin susceptible Best estimate	Global Best estimate
Isoniazid resistant*	360	1060	1420
Isoniazid susceptible*	105	8430	8540
Global	**465**	**9490**	**9960**

*All numbers rounded to significant figures

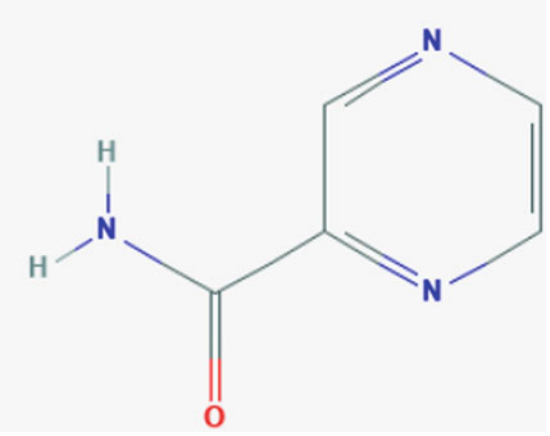


Fig. 10.4 Molecular structure of Pyrazinamide ($C_5H_5N_3O$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 1046, Pyrazinamide" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Pyrazinamide. Accessed 3 June 2021)

a mutation 'hot-spot'. The RRDR spans codons 507–533. Amino acid substitutions at codons 526 and 531 are reported to lead to high-level resistance while changes at codons 511, 516, 518, and 522 are associated with low-level resistance (Somoskovi et al. 2001). Resistance has also been associated with substitutions that occur within the *rpoB* gene, but outside the RRDR. Other mechanisms of resistance to rifampin include the permeability barrier, as well as efflux pumps, and ABC transporters (Jiang et al. 2008).

10.2.1.3 Pyrazinamide

Pyrazinamide is a synthetic pyrazinoic acid amide derivative that has bactericidal properties and is active against slowly multiplying intracellular bacilli (Fig. 10.4). Pyrazinamide or PZA is also a pro-drug that is converted into its active form of pyrazinoic acid by the enzyme nicotinamidase/pyrazinamidase (PZase) encoded by the *pncA* gene (Hazbón et al. 2006). Reported pyraziamide resistance-associated mutations are distributed along the entire length of the 561 bp *pncA* gene as well as in its promoter region. In some species of mycobacteria without *pncA* mutations, innate resistance to Pyrazinamide has been linked to a highly active pyrazinoic acid efflux mechanism (Somoskovi et al. 2001). Isolates without *pncA* mutations have also been

associated with altered pyrazinoic acid uptake and the weak binding of pyrazinoic acid to its target (Jiang et al. 2008).

10.2.1.4 Ethambutol

Ethambutol is an antibiotic with bacteriostatic, antimicrobial and antitubercular properties. Ethambutol is effective against replicating bacilli as it interferes with cell wall arabinogalactan biosynthesis (Fig. 10.5). The hypothesized target of ethambutol is the arabinosyltransferases that polymerize arabinose into arabinan (Hazbón et al. 2005). Gene transfer experiments with the *embCAB* operon, which encodes the enzymes involved in arabinogalactan biosynthesis, have provided results that suggest mutations in the *emb* operon are associated with ethambutol resistance. The most commonly occurring mutation in ethambutol-resistant *M. tuberculosis* isolates occurs within the *embB* gene (Hazbón et al. 2005). There remains about 20–35% of ethambutol-resistant isolates that do not have mutations in the *embB* gene which indicates other unknown mechanisms of ethambutol resistance do exist. An efflux pump mechanism has been associated with resistance to both ethambutol and isoniazid (Jiang et al. 2008), and occasionally resistance-conferring mutations have also been reported in *embC* (Parsons et al. 2004).

10.3 Drug Treatments

When testing samples for resistance, first-line anti-tuberculosis antimicrobials isoniazid, rifampicin, ethambutol, and pyrazinamide are the first to be tested. Second-line antimicrobials susceptibility testing can be carried out if drug resistance to first-line antimicrobials is observed (Public Health Agency of Canada 2014; Sharma et al. 2011). Public Health Agency of Canada (2014) guidelines state that the commonly used standard treatment regime for adults with fully susceptible TB disease is an initial 2-month phase of isoniazid, rifampicin, ethambutol, and potentially pyrazinamide. Ethambutol can be stopped sooner if test results determine the strain

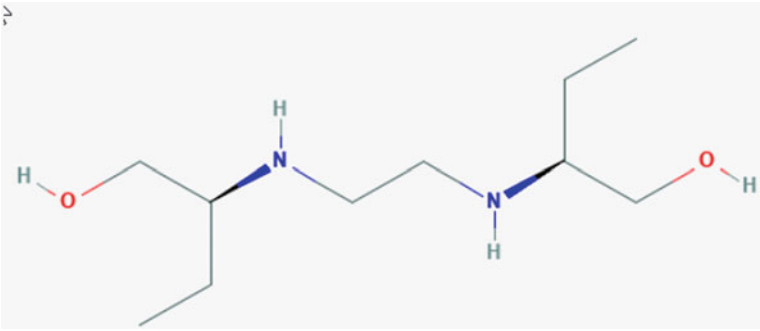


Fig. 10.5 Molecular structure of Ethambutol ($C_{10}H_{24}N_2O_2$). (National Center for Biotechnology Information. "PubChem Compound Summary for CID 14052, Ethambutol" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Ethambutol. Accessed 3 June 2021)

to be pan-sensitive. The commonly used continuation phase is administered over 4–6 months with doses of isoniazid and rifampicin. A susceptible strain is normally treated for 6–9 months and a resistant strain is normally treated for 12 to 18+ months (Gallant et al. 2017).

In Canada, resistance to isoniazid is most common (Public Health Agency of Canada 2014, 2019). In Canada, all isolates are systematically tested; strains resistant to isoniazid, or any two first-line drugs, or the multi-drug-resistant strains, are tested for second-line drugs. Treatment of drug-resistant TB often occurs with secondary antibiotics that are potentially more toxic, more expensive, and probably less effective than first-line drugs (Public Health Agency of Canada 2014). Recurrent infection can occur due to relapse with the original infection or through reinfection with a separate strain (Guerra-Assunção et al. 2014). Re-treatment cases of relapse TB are more likely to be both mono- and multi-drug resistant (MDR) than new cases (Minion et al. 2013; Dalton et al. 2012) Prior treatment with second-line drugs was a strong risk factor for resistance, and in turn increased the risk of extreme-drug resistant (XDR) TB. The second-line drugs tested included fluoroquinolones, injectables (kanamycin, amikacin, and capreomycin), and oral drugs (ethionamide and aminosalicylic acid). There are treatment guidelines in place (Falzon et al. 2011) that are attempting to reduce the accrual of drug resistance. Mixed infections occur when different strains co-infect an individual (Wang et al. 2011), and this can make diagnostics and treatment of disease more difficult (Public Health Agency of Canada 2014).

10.4 Clinical Diagnostic Tools

The primary test for clinical detection of tuberculosis is the mantoux test or tuberculin skin test, developed over 100 years ago (Davies and Pai 2008; Andersen et al. 2000). This test measures the delayed-type hypersensitivity reaction in response to TB antigens planted under the skin. A positive reaction indicates prior exposure to *M. tuberculosis*. Results are determined 48–72 h afterward by the size of swelling around the injection site (Centers for Disease Control and Prevention 2016a, b; Mayo Foundation for Medical Education and Research 2019). The sensitivity and specificity of this test is suboptimal (Andersen et al. 2000). Mantoux test shows cross-reactivity with proteins present in the Bacillus-Calmet Guerin (BCG) vaccine and with other non-tuberculous environmental *Mycobacterium* species, leading to poor test specificity (Andersen et al. 2000). The utility of this diagnostic tests is even further restricted in HIV positive and other immunocompromised patient populations as well as in children (Balcells et al. 2008; Jones et al. 1993).

Following the skin or blood test, the general next step in diagnosis is an X-ray or CT scan (Mayo Foundation for Medical Education and Research 2019). An X-ray of an individual with a TB infection can show opacities throughout the lungs, typical of pulmonary TB (U.S. National Library of Medicine 2020). While a chest X-ray cannot confirm TB diagnosis, as lesions can be indicative of other diseases, they can be used as supporting evidence of infection with laboratory results (Centers for

Disease Control and Prevention 2016a). After the clinical tests are performed, patients with suspected TB infections will usually have a sputum sample submitted to the laboratory for further testing for the presence of MTBC bacteria (Mayo Foundation for Medical Education and Research 2019).

The interferon gamma release assay is an in-vitro T-cell assay that measures the production of interferon gamma (IFN-γ) from immune cells in response to antigens specific to the RD1 (regions of deletion) region of *M. tuberculosis* that is absent from the BCG vaccine or other non-tuberculous *Mycobacterium* species (Wallis et al. 2010). White blood cells of an infected individual release interferon-γ in response to *M. tuberculosis*-derived antigens and interferon-γ release assays (IGRAs) measure this immune reactivity (Centers for Disease Control and Prevention 2016c; Mayo Foundation for Medical Education and Research 2019). The IGRA still has a limited capacity for the detection of active infection, latent case detection, false-negative test rate and restricted use in immunocompromised patients or children (Sester et al. 2011; Butera et al. 2009; Kang et al. 2005; Mahomed et al. 2006; Mori 2009). Blood IGRAs can be used in place of the Mantoux test and the test can be partly performed in hospital and a laboratory (Centers for Disease Control and Prevention 2016b).

10.5 Laboratory Diagnosis

10.5.1 Acid-Fast Bacilli Smears

Laboratory diagnosis of TB has conventionally been achieved through acid-fast bacilli (AFB) smears and culturing. AFB detection is a microscopic method which indicates the presence of acid-fast bacilli. A numbering system (1–4) is in place to quantify the bacilli seen when reporting AFB smear results. AFB smearing can utilize one of two methods: light/bright field microscopy with the traditional Ziehl-Neelsen stain, or fluorescent microscopy with an auramine stain (Christianson et al. 2013a). The Ziehl-Neelsen method utilizes a carbolfuchin stain with acid alcohol decolorizing step followed by counterstain with methylene blue counterstain. Auramine dyes (auramine-O or auramine-rhodamine) allow acid-fast bacteria to fluoresce under a fluorescent microscope (Bayot et al. 2020). Advantages of AFB smear tests are its low costs, ease of use and rapid results but disadvantages are variable, low specificity and sensitivity, sample composition, method used and subjective reporting (Public Health Agency of Canada 2014; Christianson et al. 2013a; Centers for Disease Control and Prevention 2016d; Babafemi et al. 2017; Bayot et al. 2020). A negative AFB may not necessarily rule out TB as this result could still coincide with $<$10,000 bacilli per mL of sputum (Centers for Disease Control and Prevention 2016d), as the limit of detection of AFB smears is ~10,000 organisms per mL (Lebrun et al. 1997).

10.5.2 Culture

Culturing *M. tuberculosis* is the gold standard for detecting active TB infection. It is considered the most sensitive of the conventional testing methods but is slow as results typically take 2–8 weeks (Public Health Agency of Canada 2014; Christianson et al. 2013a; Forbes et al. 2018) Culturing provides 500× the sensitivity of AFB smears and provides the added benefit of utilizing culture for further testing (Zwolska 2005). While *M. tuberculosis* can be cultured in liquid or solid media, liquid cultures have the advantage of being more rapid and sensitive, though the disadvantage of being more likely to become cross-contaminated (Cruciani et al. 2004). Culturing on solid media can take multiple weeks (Babafemi et al. 2017) for detection of growth, though some commercial broth-based systems can cut this down to 4–14 days (Centers for Disease Control and Prevention 2016d). Bactec960 MGIT, Myco-ESP culture system II, and BacT/ALERT are all automated liquid systems which are approved by Health Canada and use fluorometric or colorimetric techniques to detect culture growth. A further advantage of culture diagnosis is that this method can be performed on all specimen types (Public Health Agency of Canada 2014).

10.5.3 Drug Susceptibility Testing

Drug resistance in *M. tuberculosis* can be assayed either phenotypically or by molecular assays. Molecular methods include line-probe assays and the Xpert MTB/RIF test, along with other nucleic acid amplification methods (Public Health Agency of Canada 2014, 2019). Phenotypic assays are generally performed by incubating the infective agent with an antimicrobial on media and determining susceptibility/resistance. If an organism is unable to grow in the presence of a certain drug, it is determined to be susceptible and the drug would likely be effectively used for treatment (American Association for Clinical Chemistry 2019). Phenotypic methods can be performed on solid media which are laborious and timely, or in broth culture which are rapid and standard practice in North America (Woods et al. 2011; World Health Organization 2018). Drug susceptibility testing (DST) is normally performed for *M. tuberculosis* isolates against isoniazid, rifampicin, ethambutol, and pyrazinamide; the first-line anti-TB drugs (Centers for Disease Control and Prevention 2016d). Further DST does occur (i.e., repeat testing or second-line anti-TB drug testing) in cases in which it is warranted. Organisms may be deemed MDR if resistant to isoniazid and rifampicin. XDR-TB is determined when the organisms are resistant to isoniazid and rifampicin plus a fluoroquinolone and at least one of amikacin, kanamycin, or capreomycin (Centers for Disease Control and Prevention 2016d). DST alone cannot detect all drug resistances, prior knowledge of the mutations leading to the resistance is needed. Detection of mutations and knowledge of their significance as low-confidence or high-confidence and a quality-controlled database are extremely important. Molecular DST is normally done paired with

slow, growth-based assays as well (Sharma et al. 2011; Centers for Disease Control and Prevention 2016d).

10.5.4 Molecular Diagnostic Methods

Rapid molecular tests can be based on many principles including analysis of lipids, probe hybridization, PCR, and rRNA sequencing (Katoch 2004). Various methods of nucleic acid amplification, including PCR-based methods, can detect both the presence of MTBC and potentially drug resistance. These methods are faster than culturing methods. Commercial nucleic acid amplification assays may have high sensitivity, though are variable depending on the type of sample tested (Sarmiento et al. 2003; Ling et al. 2008a). While the sensitivity varies, the specificity of these commercial tests is ≥90% (Greco et al. 2006; Ling et al. 2008a). Commercial assays approved by Health Canada include COBAS Taqman MTB (real-time-PCR), BD ProbeTec (strand displacement amplification), Amplified MTB Direct (transcription-mediated amplification), GenoType Mycobacteria Direct (PCR) and Xpert MTB/RIF (automated cartridge-based nested PCR).

10.5.5 Xpert MTB/RIF Assay

The Xpert MTB/RIF is a cartridge-based, automated, nested, real-time PCR assay, which detects MTBC and rifampicin resistance in under 2 h (Lawn and Nicol 2011). This nucleic acid amplification-based assay mixes a sputum sample with a reagent, and the automated GeneXpert machine processes the mixture (Rachow et al. 2011). This assay is 98% specific, 85% sensitive, and rapid (Rachow et al. 2011; Steingart et al. 2013; Li et al. 2017). Advantages of this system include the very short amount of required hands-on work and the limited user infection risk due to inactivation via reagents used (Banada et al. 2010). Another benefit is that this test can be used on direct sputum samples. As opposed to AFB smearing, Xpert MTB/RIF assays have the ability to accurately differentiate between non-tuberculous mycobacteria (NTM) and MTBC (Steingart et al. 2013). The Xpert MTB/RIF assay does, however, detect both live and dead bacteria (Miotto et al. 2012). The Public Health Agency of Canada (2019) recommends that Xpert results should still be confirmed with culturing to rule out the possibility of a falsely-positive rifampicin-resistant result. Helb et al. (2009) found that the limit of detection for *M. tuberculosis* with Xpert was 4.5 genomes per reaction when DNA was used, and 131 cfu/mL when spiked sputum was tested. These authors (Helb et al. 2009) found that 23 common rifampicin resistance mutations could be detected with this assay and that after correcting for a 'conventional susceptibility test error', all susceptible samples were determined as

such. Armand et al. (2011) also found that the Xpert assay had better sensitivity for respiratory samples.

10.5.6 Line Probe Assays

Line probe assays (LPAs), such as the GenoType MTBDRplus LPA assay, are another molecular method of DST, developed for use with smear-positive sputum samples and culture isolates (Public Health Agency of Canada 2014). LPAs have a limit of detection of 10,000 cfu/mL (Ninan et al. 2016). This is a disadvantage when compared to Xpert MTB/RIF, as fewer samples may be detected with LPAs. The GenoType MTBDRplus LPA assay has been shown to have high sensitivity and specificity (>98%), though isoniazid resistance/sensitivity was inconsistent (Ling et al. 2008b). The World Health Organization has supported the use of LPAs for detection of resistance to rifampicin and isoniazid from sputum samples (World Health Organization 2008) based on detecting mutations in *rpoB* (rifampicin), *katG* (isoniazid), and *inhA* (isoniazid) genes. Brossier et al. (2010) found that the sensitivity for isoniazid resistance detection increased from 67% with the MTBDR to 86% with MTBDRplus. The GenoType MTBDR assay detects mutations only in *rpoB* and *katG*, not *inhA*, to determine resistance to rifampicin and isoniazid (Bang et al. 2006). Another variation, the GenoType MTBDR*sl*, can detect resistance in *M. tuberculosis* to ethambutol, fluoroquinolone, streptomycin, amikacin, kanamycin, and capreomycin. Fluoroquinolone resistance is detected by mutations in *gyrA* and *gyrB*, streptomycin resistance in *rpsL*, aminoglycoside/cyclic peptide resistance in *rrs* and *tlyA*, and ethambutol resistance in *embB*. This test can therefore be satisfactory for these given mutations but again, can miss mutations (which ultimately lead to resistance) in other genes or gene segments (Brossier et al. 2010).

LiPA is another LPA that can detect rifampicin resistance only, with high sensitivity (ranging 82 to 100%) and specificity (ranging 92–100%) from culture, though the sensitivity decreases with direct clinical samples. (Morgan et al. 2005).

10.5.7 PCR-Based Methods

Real-time polymerase chain reaction assay (RT-PCR), which combines PCR with fluorescent probe detection, is generally faster than conventional PCR and is comparable in sensitivity and specificity (Espy et al. 2006; Babafemi et al. 2017), but like other molecular assays, does not distinguish between viable and dead bacteria (Kralik and Ricchi 2017). While smear microscopy requires 5000–10,000 organisms per mL, RT-PCR only needs around six copies of DNA per mL (Babafemi et al. 2017). Other advantages of RT-PCR are the speed to results with accuracy and the fact that bacterial load can be quantified. Further, because this method can be automated, the required hands-on time as well as the cross-contamination risk are reduced (Katoch 2004; Sethi et al. 2012). Babafemi et al. (2017) note that this should not be used as a stand-alone assay but in support of conventional assays. Sethi et al.

(2012) found that the *mpt64* RT-PCR assay had a higher sensitivity than IS*6110* PCR. Zakham et al. (2012) found that PCR using IS*6110* had sensitivity and specificity of 92% and 98%, respectively. Copy numbers and insertion positions are variable between different strains (Thorne et al. 2011), making this an adequate marker for phylogenetic analysis and epidemiology. Choi et al. (2015) used the 16S rRNA sequence as a PCR target, differing from the commonly used IS*6110* sequence. Results of this study suggest that the 16S sequence is comparable as a PCR target to IS*6100* for *M. tuberculosis* detection with high sensitivity. 16S rRNA PCR can be a useful tool but quality databases are essential (Böttger 1989).

Warren et al. (2004) and Wang et al. (2011) developed PCR methods to detect mixed *M. tuberculosis* infections, with high sensitivity and specificity. Warren et al. (2004) noted that mixed infections were more common in cases of re-treatment. Woods et al. (2011) remind that resistance caused by mutations that are not detected by these molecular methods due to their design may still occur and as such, these tests are not perfect; interpretation of results needs to consider this fact. On the positive side, these molecular methods greatly reduce the time to results compared to culturing (i.e., 1 month versus 1 day) (Woods et al. 2011). Rapid drug-resistance results allow an earlier start of effective therapy, which leads to better outcomes for patients, overall public health, and epidemiology.

10.5.8 Genotyping

Genotyping of *M. tuberculosis* from culture is a slow and laborious process. While restriction fragment length polymorphism or spoligotyping or mycobacterial interspersed repetitive units may be methods of TB genotyping, the application of whole-genome sequencing (WGS) allows for *M. tuberculosis* identification, drug resistance prediction, in-depth investigation of strains and their genetic relatedness by use of single assay (Gardy et al. 2011; Walker et al. 2013; Cowan and Crawford 2002; Tyler et al. 2016, 2017; Pankhurst et al. 2016; Christianson et al. 2013a, b; Cowan et al. 2012; Sharma 2011). Sequence data generated from WGS can discriminate between closely related outbreaks that descend from a historical common/recent ancestor and provide inference for the direction of transmission within outbreaks. In addition, the integration of WGS with epidemiological information can identify transmission events and the presence of super-spreaders. Pankhurst et al. (Pankhurst et al. 2016) identified outbreaks that were missed by conventional methods, and demonstrated that the use of WGS for surveillance and outbreak investigations can better identify the chain of TB transmission networks. Culture-independent WGS from samples rather than cultures have the potential to expedite the reporting process by approximately 2 months. Tuberculosis is endemic in select vulnerable populations with rates of TB far exceeding the Canadian average (Patel et al. 2017; Tyler et al. 2017). The transmission of small-cluster long-term outbreaks, by use of genotyping, are being identified on an ongoing basis. Lack of infrastructure, geographic isolation, rapid tests and scarce human resources have challenged the ability of local public health officials to effectively monitor and control TB

spread (Gardy et al. 2011; Christianson et al. 2013b; Lee et al. 2015; Patel et al. 2017; Tyler et al. 2017). Newer technologies to investigate the outbreaks have emerged and evolved to help better understand the dynamics of TB transmission (Gardy et al. 2011; Walker et al. 2013; Tyler et al. 2017; Pankhurst et al. 2016). This is essential to allow public health officials to more efficiently and effectively address the transmission and spread of TB.

10.6 Whole-Genome-Sequencing-Based Advanced Diagnostics and Research

Molecular methods have advantages over conventional testing methods including the gold standard of culture, though what can be tested is limited (Bryant et al. 2015). The capability to show the gain or loss of resistance genes is not particularly relevant for TB as HGT does not occur in MTBC (Tamma et al. 2018). TB resistance generally occurs through point mutations. WGS is an appropriate method of testing for resistance as well as lineage, with the further advantage that it can be determined through a single procedure. Faster diagnosis, with accurate predictions of resistance, identification of novel mutations as well as being financially feasible are all further advantages of WGS (Walker et al. 2015; Farhat et al. 2016; Pankhurst et al. 2016; Miotto et al. 2012; Allix-Béguec et al. 2018; Zignol et al. 2018). These can lead to positive implications regarding patient networks and therapy regimes (Witney et al. 2016). WGS has some disadvantages: potential increased costs during the transition period, knowledge needed for interpretation of results, the genotype-phenotype relationship of novel markers, and presence of standards that exist for newer technologies (Rodwell 2019).

Chen et al. (2019) found that WGS could be used to predict isolate resistance to isoniazid, rifampicin, pyrazinamide, levofloxacin, amikacin, kanamycin, capreomycin, streptomycin, and prothionamide and accuracy, sensitivity, and specificity of these predictions were >85%. These authors found that the determined WGS results were overall consistent with DST results. WGS can be useful in predicting drug resistance of an isolate as well as in relating isolates for analysis of transmission and outbreaks through genotyping: the process of determining sequence variations to determine associations (Bryant et al. 2013; Roetzer et al. 2013; Walker et al. 2013; Witney et al. 2016; Integrated DNA Technologies 2020). Witney et al. (2016) note that appropriate software and bioinformatic tools are required for the ideal use of WGS methods. The costs of performing WGS versus routine DST are comparable, and so after preliminary validation, implementation is possible without increased routine costs (Witney et al. 2016).

10.6.1 Illumina and Oxford Nanopore MinION

Illumina sequencing occurs through sequencing by synthesis. Fluorescently labelled dNTPs are incorporated into DNA fragments in a massively parallel fashion.

Illumina sequencing includes four steps: library preparation by fragmentation and adapter ligation, cluster generation with adapters binding a flow cell, sequencing, and finally data analysis and alignment to a reference genome (Illumina 2017).

Recently, Oxford Nanopore sequencing has been piquing interest. Deamer et al. (2016) document the history of the development of nanopore sequencing. Oxford Nanopore's MinION sequencer, released in 2014 (Lu et al. 2016) utilizes nanopore technology to sequence DNA. Nanopore sequencing is fast, sensitive, and produces long read length WGS (Jain et al. 2015). Nanopore MinION sequencing occurs as a DNA strand is threaded through a protein pore with applied electrical current. The current flowing through the pore changes depending on which base is passing through it at any given time. This continuous change in current is used to determine the DNA sequence of the strand (Schürch and Schaik 2017; Tamma et al. 2018). This sequencing platform has an error rate between 5 and 20% (Kono and Arakawa 2019), though this issue can be accounted for by error correction and assembly strategies (Goodwin et al. 2015; Jain et al. 2015; Loman et al. 2015; Leggett et al. 2015; Schürch and Schaik 2017).

The initial investment for MinION is approximately Canadian $1000 for a basic package which includes two flow cells and a starter kit of reagents. An additional kit costs $650 for 12 samples for PCR and barcoding kit or $599 for $1D^2$ sequencing kit (R9.5). A large capital investment is required for sequencing platforms which can range from $50,000 to $100,000 for desktop sequencers, and 10× more for high-throughput platforms. MiSeq materials cost including culture and labour for sequencing is approximately $200/sample; depending on urgent (3 samples) or non-urgent submission (12 samples), and fresh or stocked culture. The type of extraction kit used will also alter the cost (Tyler et al. 2017; Brown et al. 2015; Tyler et al. 2016; Pankhurst et al. 2016).

MinION also has the advantage of real-time data analysis; the sequencing data can be analyzed as it is being produced (Judge et al. 2015; Schürch and Schaik 2017; Cao et al. 2016; Tamma et al. 2018). This allows strain identification, drug resistance detection and genotype in very little time. For slow-growing organisms like *M. tuberculosis,* this is a huge advantage (Schürch and Schaik 2017). Pankhurst et al. (2016) showed that DST prediction via WGS was sufficiently accurate (93%) while also being faster and more cost-effective than culturing methods. Illumina sequencing platforms produce reads which may not be long enough to cover various repeat elements in a bacterial genome while MinION, on the other hand, is a long-read platform, allowing more complete and greater quality bacterial genome assembly (Lu et al. 2016; Schürch and Schaik 2017). The production of long reads by nanopore sequencing helps to assemble a genome, specifically through areas with repetition and 'structural variations' potentially including indels, duplications, or inversions (Tamma et al. 2018). Bainomugisa et al. (2018) were able to assemble an isolate genome with 99.92% accuracy using only MinION reads. When Illumina reads were used to complement this data, accuracy was 99.98%. This assembly used 238x coverage and the estimated error rate of MinION data was 5.3%. Furthermore, this platform is portable due to its small size, is relatively affordable, and can quickly produce data in real-time (Lu et al. 2016; Schmidt et al. 2016). The small size and

portability of the MinION sequencer give this platform good potential for use outside of clinical laboratories (Schürch and Schaik 2017).

This platform also has some downfalls. SNPs in DNA, which can have greater effects on the protein level, may be difficult to distinguish with MinION technologies (Tamma et al. 2018). While random error should generally be removed during assembly, any systematic errors may not be as well accounted for, such as homopolymers or methylation (methylation can affect the electrical signal utilized for nanopore sequencing, which can lead to errors) (Tamma et al. 2018). The use of a method for WGS depends on urgency or the type of result needed.

10.6.2 WGS-Based Diagnostic Methods Directly from Sputum Samples

Previous studies have resulted in poor outcomes when performing WGS directly from respiratory samples but improvements by targeted enrichment via use of oligonucleotide beads to capture *M. tuberculosis* DNA prior to WGS has resulted in >20× coverage and >98% mapped genome in 83% of the study isolates (Brown et al. 2015). Developing a culture-independent diagnostic test that circumvents the lengthy 2–8-week culture step will undoubtedly lead to significant improvements in turnaround time for the *M. tuberculosis* laboratory program. However, limitations for direct WGS from sputa samples include, low bacterial load to host DNA ratio, potential *M. tuberculosis* infections, and other background microbiota that add additional challenges which will need to be addressed through both wet- and dry-laboratory strategies.

Votintseva et al. (2017) developed a method for *M. tuberculosis* DNA extraction directly from respiratory samples, a method which does not include a sample enrichment step. This allows the potential for the time to results to be less than a day when using platforms such as MinION. Votintseva et al. (2017) tested their extraction protocol with three sequencers: Illumina MiSeq, Illumina MiniSeq, and Oxford Nanopore MinION. A depth of >3× was needed for resistance predictions. 96% of the predictions made agreed with the results of DST, those that did not were a patient with a mixed infection with different resistance patterns. Brown et al. (2015) used biotinylated RNA bait to gather *M. tuberculosis* DNA from direct sputum samples. Being able to gather DNA directly from sputum allows the advantage of reduced time to diagnosis. The RNA oligo baits spanned the sequence of the entire *M. tuberculosis* genome. Once captured, DNA was then amplified and sequenced. The resulting reads had adequate depth and accurately predicted mutations associated with drug resistance (Witney et al. 2016). This method was also able to predict the presence of a mixed infection. Of the smear-positive samples, >98% of the *M. tuberculosis* genome was able to be recovered with this method in 83% of samples. For most samples, the genome coverage and depth were comparable to matched cultures. In the cases with lower coverages, it was hypothesized that these results were due to low pathogen load. The resistance predictions, compared to culture vs sputum, were in agreement. Doyle et al. (2018) evaluated WGS from

sputum samples in which mixed infections were present, noting that MGIT and solid culture do not identify these cases well (Martin et al. 2010; Hanekom et al. 2013). The authors were able to detect SNPs consistent with mixed infections in patient samples.

The challenges that are faced when trying to sequence DNA directly from sputum samples are; (a) the low amount of actual desired organisms and (b) these samples also contain DNA from cells of host, NTM and other microbes (Iketleng et al. 2018). Methods to enrich for target DNA during extraction steps can be utilized to optimize WGS results from sputum, such as Votintseva et al. (2017). The presence of different DNA sources also ultimately leads to a reduced sequencing depth of the desired genome (Doughty et al. 2014). This is particularly an issue for drug resistance detection. The advantage of real-time sequencing with MinION is that sequencing can continue until the desired coverage is reached, hence saves time when DNA load is higher and allowing continuation when it is lower. Votintseva et al. (2017) found that no false-positive resistance predictions were made, though authors did note that in order for all mutations in their catalogue to be identified, deep coverage was required. They also found systematic SNP error biases: an A to G error bias, though this could be corrected for in mapping. Even with higher error rates, deep coverage could lead to accurate resistance profiles. While rapid molecular tests can provide some information on drug resistance, WGS can provide a complete genetic profile. WGS can be a slower process but can be sped up when DNA is sequenced from sputum samples directly versus from cultured isolates. The issue with these direct samples is that the DNA can often be of poor quality and in low concentrations. This can be somewhat improved upon when a step to deplete undesired (e.g., human) DNA is included in the process. Doughty et al. (2014) used shotgun metagenomic sequencing on sputum samples with an Illumina MiSeq platform to detect *M. tuberculosis*. Their method, lacking any culturing, capturing, or amplification steps, did not provide sufficient coverage to determine drug resistance.

The large amounts of data that can be produced by WGS can lead to challenges being faced. These include methods for storing and analyzing these data as well as requiring specialized staff with the skill to analyze the data (Iketleng et al. 2018). Further limitations include needing enrichment steps, capable staff and equipment, and adequate bioinformatic systems (Lee and Behr 2015). Standardization is also difficult for assays on direct sputum samples (Lee and Behr 2015).

10.6.3 Bioinformatic Tools

There are many tools that have been developed for data analysis and error correction of WGS data. These include TB Profiler, Mykrobe Predictor TB, CASTB, KvarQ, and PhyResSE (Schleusener et al. 2017). Cao et al. (2016) developed a streaming algorithm and pipeline which can process 100× more data than the MinION computer throughput. Oxford Nanopore's Metrichor has platforms including the EPI2ME platform. This cloud-based platform allows analysis of data in real-time (Metrichor 2020). Greninger et al. (2015) developed a web-based pipeline,

MetaPORE, which couples with MinION to perform real-time analysis. MetaPORE is also a visualization tool and can identify a pathogen from Nanopore data. Raw Nanopore data is base-called using a Metrichor pipeline (Metrichor 2D Basecalling v1.14 pipeline) which is then scanned for sequence reads to subsequently be aligned using BLASTn/MegaBLAST. This can process the data to a result which corresponds to an identification. The reference sequence is determined by having the best match at each refresh, and maps are generated based on alignment. It's advantage is that it is web-based and can run on a regular laptop. Ellington et al. (2017) advocate that a single database should house all resistance information to ease comparison, updating, and curation. The Mykrobe predictor tool compares De Bruijn graphs to determine species diagnosis and predict drug resistance. This tool is able to analyze read data as is produced from MinION sequencing, and can account for multiple bacteria being present in a sample (Schürch and Schaik 2017). Bradley et al. (2015) found that sensitivity and specificity of resistance of *M. tuberculosis* using the Mykrobe predictor tool was 82.6% and 98.5%, respectively. Goodwin et al. (2015) developed Nanocorr, an error correction algorithm for Nanopore, specifically. This algorithm can handle the longer read lengths of 5–50 kb with higher error rates of 5–40%. The authors used Nanopore reads supplemented with Illumina MiSeq reads to sequence a bacterial genome. It is suggested that this method is superior to MiSeq data alone as some genomic features (rRNA, transposable elements, etc.) were better represented. Jain et al. (2015) used a tool to find SNPs and maxlik estimates. Loman et al. (2015) assembled an *E. coli* genome with Nanopore data with a three-staged method in which they detected read overlaps, corrected reads, and used a probabilistic model to 'polish' the assembly. This method realized nucleotide identity of 99.5%. The online TB Profiler tool allows reporting of drug resistance from raw sequences (Coll et al. 2015). Many other in-house developed methods and tools are also in use. Quality control, verification and validation of these tools and platforms should be done before implementing in a routine clinical laboratory (Forbes et al. 2018).

10.7 Conclusion

The causative agent of TB, *M. tuberculosis* is a slowing-growing bacteria and the methods used for identification, susceptibility testing and genotyping are primarily culture-based including liquid media culturing and susceptibility testing, 16S, *hsp65*, resistance marker gene sequencing, MIRU, spoligotyping, and restriction fragment length-based polymorphism, etc. Timely identification, treatment and surveillance of *M. tuberculosis* is hindered by the inability to rapidly and accurately identify, characterize and genotype strains directly from patient samples. This has been challenged in recent years due to SARS CoV-2 infections and outbreaks. The potential to diagnose TB, predict resistance and delineate transmission networks through the use of a single test, such as WGS, has the capability to enhance TB laboratory, TB control and prevention programs. Quality assessment of generated

sequence data, testing and validation of assays and bioinformatics pipeline parameters are all needed prior to implementation.

References

Allix-Béguec C, Arandjelovic I, Bi L, Beckert P, Bonnet M, Bradley P, Cabibbe AM, Cancino-Muñoz I, Caulfield MJ, other authors. (2018) Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing. N Engl J Med 379:1403–1415

American Association for Clinical Chemistry (2019) Antibiotic susceptibility testing [online]. https://labtestsonline.org/tests/antibiotic-susceptibility-testing. Accessed 14 July 2020

Andersen P, Munk ME, Pollock JM, Doherty TM (2000) Specific immune-based diagnosis of tuberculosis. Lancet 356:1099–1104

Armand S, Vanhuls P, Delcroix G, Courcol R, Lemaitre N (2011) Comparison of the Xpert MTB/RIF test with an IS*6110*-TaqMan real-time PCR assay for direct detection of *Mycobacterium tuberculosis* in respiratory and nonrespiratory specimens. J Clin Microbiol 49:1772–1776

Babafemi EO, Cherian BP, Banting L, Mills GA, Ngianga K (2017) Effectiveness of real-time polymerase chain reaction assay for the detection of *Mycobacterium tuberculosis* in pathological samples: a systematic review and meta-analysis. Syst Rev 6:215

Bainomugisa A, Duarte T, Lavu E, Pandey S, Coulter C, Marais BJ, Coin LM (2018) A complete high-quality MinION nanopore assembly of an extensively drug-resistant *Mycobacterium tuberculosis* Beijing lineage strain identifies novel variation in repetitive PE/PPE gene regions. Microb Genomics 4:e000188

Balcells ME, Perez CM, Chanqueo L et al (2008) A comparative study of two different methods for the detection of latent tuberculosis in HIV-positive individuals in Chile. Int J Infect Dis 12(6): 645–652

Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U (2012) Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates. Antimicrob Agents Chemother 56:2643–2651

Banada PP, Sivasubramani SK, Blakemore R, Boehme C, Perkins MD, Fennelly K, Alland D (2010) Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. J Clin Microbiol 48:3551–3557

Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G, Jacobs W (1994) inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science 263:227–230

Bang D, Andersen AB, Thomsen VO (2006) Rapid genotypic detection of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* directly in clinical specimens. J Clin Microbiol 44:2605–2608

Bayot ML, Mirza TM, Sharma S (2020) Acid fast bacteria. In: StatPearls [Internet]. StatPearls Publishing, Treasure Island

Blanchard JS (1996) Molecular mechanisms of drug resistance in Mycobacterium tuberculosis. Annu Rev Biochem 65:215–239

Böttger EC (1989) Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol Lett 65:171–176

Bradley P, Gordon NC, Walker TM, Dunn L, Heys S, Huang B, Earle S, Pankhurst LJ, Anson L et al (2015) Rapid antibiotic-resistance predictions from genome sequence data for *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Nat Commun 6:10063

Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W (2010) Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 48:1683–1689

Brown AC, Bryant JM, Einer-Jensen K, Holdstock J, Houniet DT, Chan JZM, Depledge DP, Nikolayevskyy V, Broda A et al (2015) Rapid whole-genome sequencing of *Mycobacterium tuberculosis* isolates directly from clinical samples. J Clin Microbiol 53:2230–2237

Bryant JM, Schürch AC, Deutekom HV, Harris SR, Beer JLD, Jager VD, Kremer K, Van Hijum SAFT, Siezen RJ et al (2013) Inferring patient to patient transmission of *Mycobacterium tuberculosis* from whole genome sequencing data. BMC Infect Dis 13:110

Bryant JM, Lipman M, Breuer J (2015) Personalizing therapy for multidrug resistant TB: the potential of Rapid Whole Genome Sequencing. Expert Rev Anti-Infect Ther 14:1–3

Butera O, Chiacchio T, Carrara S et al (2009) New tools for detecting latent tuberculosis infection: evaluation of RD1-specific long-term response. BMC Infect Dis 9:182

Cao MD, Ganesamoorthy D, Elliott AG, Zhang H, Cooper MA, Coin LJ (2016) Streaming algorithms for identification of pathogens and antibiotic resistance potential from real-time MinION sequencing. GigaScience 5:32

Centers for Disease Control and Prevention (2016a) Diagnosis of tuberculosis disease [online]. https://www.cdc.gov/tb/publications/factsheets/testing/diagnosis.htm. Accessed 29 June 2020

Centers for Disease Control and Prevention (2016b) Testing for TB infection [online]. https://www.cdc.gov/tb/topic/testing/tbtesttypes.htm. Accessed 29 June 2020

Centers for Disease Control and Prevention (2016c) Interferon-gamma release assays (IGRAs)—blood tests for TB infection [online]. https://www.cdc.gov/tb/publications/factsheets/testing/igra.htm#:~:text=Interferon%2DGamma%20Release%20Assays%20(IGRAs)%20are%20whole%2Dblood,(LTBI)%20from%20tuberculosis%20disease. Accessed 29 June 2020

Centers for Disease Control and Prevention (2016d) Diagnosis of tuberculosis disease. In: Core curriculum on TB. Centers for Disease Control and Prevention. https://www.cdc.gov/TB/education/corecurr/pdf/chapter4.pdf

Chen X, He G, Wang S, Lin S, Chen J, Zhang W (2019) Evaluation of whole-genome sequence method to diagnose resistance of 13 anti-tuberculosis drugs and characterize resistance genes in clinical multi-drug resistance *Mycobacterium tuberculosis* isolates from China. Front Microbiol 10:1741

Choi Y, Hong S-R, Jeon B-Y, Wang H-Y, Lee G-S, Cho S-N, Shim TS, Lee H (2015) Conventional and real-time PCR targeting 16S ribosomal RNA for the detection of *Mycobacterium tuberculosis* complex. Int J Tuberc Lung Dis 19:1102–1108

Christianson S, Jamieson F, Wolfe J, Sharma M (2013a) Appendix D: Tuberculosis and mycobacteriology laboratory standards: services and policies. In: Canadian tuberculosis standards, 7th edn. Public Health Agency of Canada and the Canadian Lung Association/Canadian Thoracic Society

Christianson S, Sharma MK, Baikie M, Randell E, Wolfe J (2013b) Molecular epidemiology of tuberculosis in the Nunavut territory in Canada. Int J Circumpolar Health 72:22447. https://doi.org/10.3402/ijch.v72i0.22447

Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbón MH, Harbacheuski R, Megjugorac NJ, Jacobs WR, Holzenburg A (2005) The Mycobacterium tuberculosis iniA gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. Mol Microbiol 55:1829–1840

Coll F, Mcnerney R, Preston MD, Guerra-Assunção JA, Warry A, Hill-Cawthorne G, Mallard K, Nair M, Miranda A et al (2015) Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med 7:51

Cowan L, Crawford J (2002) National tuberculosis genotyping and surveillance network: analysis of the genotype database. Emerg Infect Dis 8(11):1294–1302

Cowan LS, Hooks DP, Christianson S, Sharma MK, Alexander DC, Guthrie JL, Jamieson FB, Supply P, Allix-Béguec C, Cruz L, Desmond E, Kramer R, Lugo S, Rudrik J (2012) Evaluation of mycobacterial interspersed repetitive-unit-variable-number tandem-repeat genotyping as performed in laboratories in Canada, France, and the United States. J Clin Microbiol 50(5):1830–1831; author reply 1832

Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C (2004) Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of Mycobacteria. J Clin Microbiol 42:2321–2325

Dalton T, Cegielski P, Akksilp S, Asencios L, Caoili JC, Cho S-N, Erokhin VV, Ershova J, Gler MT, other authors. (2012) Prevalence of and risk factors for resistance to second-line drugs in people with multidrug-resistant tuberculosis in eight countries: a prospective cohort study. Lancet 380:1406–1417

Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433

Davies PD, Pai M (2008) The diagnosis and misdiagnosis of tuberculosis. Int J Tuberc Lung Dis 12: 1226–1234

Deamer D, Akeson M, Branton D (2016) Three decades of nanopore sequencing. Nat Biotechnol 34:518–524

Doughty EL, Sergeant MJ, Adetifa I, Antonio M, Pallen MJ (2014) Culture-independent detection and characterisation of *Mycobacterium tuberculosis* and *M. africanumin* sputum samples using shotgun metagenomics on a benchtop sequencer. PeerJ 2:e585

Doyle RM, Burgess C, Williams R, Gorton R, Booth H, Brown J, Bryant JM, Chan J, Creer D et al (2018) Direct whole-genome sequencing of sputum accurately identifies drug-resistant *Mycobacterium tuberculosis* faster than MGIT culture sequencing. J Clin Microbiol 56:e00666–e00618

Eldholm V, Balloux F (2016) Antimicrobial resistance in *Mycobacterium tuberculosis*: the odd one out. Trends Microbiol 24:637–648

Eldholm V, Norheim G, Lippe BVD, Kinander W, Dahle UR, Caugant DA, Mannsåker T, Mengshoel AT, Dyrhol-Riise AM, Balloux F (2014) Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. Genome Biol 15: 490

Ellington M, Ekelund O, Aarestrup F, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden M et al (2017) The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect 23:2–22

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JDC, Wengenack NL, Rosenblatt JE et al (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165–256

Falzon D, Jaramillo E, Schunemann HJ, Arentz M, Bauer M, Bayona J, Blanc L, Caminero JA, Daley CL et al (2011) WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. Eur Respir J 38:516–528

Farhat MR, Sultana R, Iartchouk O, Bozeman S, Galagan J, Sisk P, Stolte C, Nebenzahl-Guimaraes H, Jacobson K et al (2016) Genetic determinants of drug resistance in *Mycobacterium tuberculosis* and their diagnostic value. Am J Respir Crit Care Med 194:621–630

Forbes B, Miller M, Banaei N, Brown-Elliot B, Das S, Salfinger M, Sharma MK, Somoskovi A, Tans-Kersten J, Tenoer FC, Warshauer D, Zelazny AM (2018) M48—laboratory detection and identification of mycobacteria, 2nd edn. Clinical and Laboratory Standards Institute

Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, Johnston JC, Gardy J, Lipsitch M, Fortune SM (2013) *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet 45:784–790

Gallant V, Vachon J, Siu W (2017) Tuberculosis drug resistance in Canada: 2006—2016. Can Commun Dis Rep 43:236–241

Gardy J, Johnston J, Sui S, Cook V, Shah L, Brodkin E, Rempel S, Moore R, Zhao Y, Holt R, Varhol R, Birol I, Lem M, Sharma MK, Elwood K, Jones S, Brinkman F, Brunham R, Tang P (2011) Whole genome sequencing and social network analysis of a tuberculosis outbreak. N Engl J Med 364:730–739

Goodwin S, Gurtowski J, Ethe-Sayers S, Deshpande P, Schatz MC, Mccombie WR (2015) Oxford Nanopore sequencing, hybrid error correction, and de novo assembly of a eukaryotic genome. Genome Res 25:1750–1756

Greco S, Girardi E, Navarra A, Saltini C (2006) Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. Thorax 61:783–790

Greninger AL, Naccache SN, Federman S, Yu G, Mbala P, Bres V, Stryke D, Bouquet J, Somasekar S et al (2015) Rapid metagenomic identification of viral pathogens in clinical samples by real-time nanopore sequencing analysis. Genome Med 7:99

Guerra-Assunção JA, Houben RMGJ, Crampin AC, Mzembe T, Mallard K, Coll F, Khan P, Banda L, Chiwaya A, other authors. (2014) Recurrence due to relapse or reinfection with *Mycobacterium tuberculosis*: a Whole-genome sequencing approach in a large, population-based cohort with a high HIV infection prevalence and active follow-up. J Infect Dis 211:1154–1163

Hanekom M, Streicher EM, Berg DVD, Cox H, Mcdermid C, Bosman M, Pittius NCGV, Victor TC, Kidd M et al (2013) Population structure of mixed *Mycobacterium tuberculosis* infection is strain genotype and culture medium dependent. PLoS One 8:e70178

Hazbón MH, del Valle MB, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, Colangeli R, Safi H, Billman-Jacobe H, Lavender C (2005) Role of embB codon 306 mutations in Mycobacterium tuberculosis revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. Antimicrob Agents Chemother 49:3794–3802

Hazbón MH, Brimacombe M, del Valle MB, Cavatore M, Guerrero MI, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L (2006) Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 50:2640–2649

Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, Kop J, Owens MR, Rodgers R, other authors. (2009) Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol 48:229–237

Iketleng T, Lessells R, Dlamini MT, Mogashoa T, Mupfumi L, Moyo S, Gaseitsiwe S, Oliveira TD (2018) *Mycobacterium tuberculosis* next-generation whole genome sequencing: opportunities and challenges. Tuberc Res Treat 2018:1–8

Illumina (2017) An introduction to next-generation sequencing technology [online]. https://www.illumina.com/science/technology/next-generation-sequencing.html. Accessed 15 July 2020

Integrated DNA Technologies (2020) Understanding genotyping [online]. https://www.idtdna.com/pages/applications/genotyping. Accessed 15 July 2020

Jain M, Fiddes IT, Miga KH, Olsen HE, Paten B, Akeson M (2015) Improved data analysis for the MinION nanopore sequencer. Nat Methods 12:351–356

Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, Wang H (2008) Assessment of efflux pump gene expression in a clinical isolate Mycobacterium tuberculosis by real-time reverse transcription PCR. Microb Drug Resist 14:7–11

Jones BE, Young SM, Antoniskis D, Davidson PT, Kramer F, Barnes PF (1993) Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. Am Rev Respir Dis 148:1292–1297

Judge K, Harris SR, Reuter S, Parkhill J, Peacock SJ (2015) Early insights into the potential of the Oxford Nanopore MinION for the detection of antimicrobial resistance genes. J Antimicrob Chemother 70:2775–2778

Kang YA, Lee HW, Yoon HI et al (2005) Discrepancy between the tuberculin skin test and the whole-blood interferon gamma assay for the diagnosis of latent tuberculosis infection in an intermediate tuberculosis-burden country. JAMA 293:2756–2761

Katoch VM (2004) Newer diagnostic techniques for tuberculosis. Indian J Med Res 120:418–428

Kono N, Arakawa K (2019) Nanopore sequencing: review of potential applications in functional genomics. Develop Growth Differ 61:316–326

Kralik P, Ricchi M (2017) A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. Front Microbiol 8:108

LaFreniere M, Hussain H, Vachon J (2018) Tuberculosis drug resistance in Canada: 2017. Can Commun Dis Rep 44:290–296

Lawn SD, Nicol MP (2011) Xpert®MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol 6:1067–1082

Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, Vlieghe E, Hara GL, Gould IM et al (2013) Antibiotic resistance—the need for global solutions. Lancet Infect Dis 13:1057–1098

Lebrun L, Mathieu D, Saulnier C, Nordmann P (1997) Limits of commercial molecular tests for diagnosis of pulmonary tuberculosis. Eur Respir J 10:1874–1876

Lee RS, Behr MA (2015) The implications of whole-genome sequencing in the control of tuberculosis. Ther Adv Infect Dis 3:47–62

Lee RS, Radomski N, Proulx J-F, Levade I, Shapiro BJ, McIntosh F, Soualhine H, Menzies D, Behr MA (2015) Population genomics of Mycobacterium tuberculosis in the Inuit. Proc Natl Acad Sci 112(44):13609–13614. https://doi.org/10.1073/pnas.1507071112

Leggett RM, Heavens D, Caccamo M, Clark MD, Davey RP (2015) NanoOK: multi-reference alignment analysis of nanopore sequencing data, quality and error profiles. Bioinformatics 32(1):142–144

Li S, Liu B, Peng M, Chen M, Yin W, Tang H, Luo Y, Hu P, Ren H (2017) Diagnostic accuracy of Xpert MTB/RIF for tuberculosis detection in different regions with different endemic burden: a systematic review and meta-analysis. PLoS One 12:e0180725

Ling DI, Flores LL, Riley LW, Pai M (2008a) Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. PLoS One 3:e1536

Ling DI, Zwerling AA, Pai M (2008b) GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. Eur Respir J 32:1165–1174

Loman NJ, Quick J, Simpson JT (2015) A complete bacterial genome assembled de novo using only nanopore sequencing data. Nat Methods 12:733–735

Lu H, Giordano F, Ning Z (2016) Oxford nanopore MinION sequencing and genome assembly. Genomics Proteomics Bioinformatics 14:265–279

Mahomed H, Hughes EJ, Hawkridge T et al (2006) Comparison of mantoux skin test with three generations of a whole blood IFN-gamma assay for tuberculosis infection. Int J Tuberc Lung Dis 10:310–316

Martin A, Herranz M, Serrano MJR, Bouza E, Viedma DGD (2010) The clonal composition of *Mycobacterium tuberculosis* in clinical specimens could be modified by culture. Tuberculosis 90:201–207

Mayo Foundation for Medical Education and Research (2019) Tuberculosis [online]. https://www.mayoclinic.org/diseases-conditions/tuberculosis/diagnosis-treatment/drc-20351256#:~:text=Sputum%20tests,drug%2Dresistant%20strains%20of%20TB. Accessed 29 June 2020

Metrichor (2020) About Metrichor [online]. https://metrichor.com/technology.html#. Accessed 1 June 2020

Minion J, Gallant V, Wolfe J, Jamieson F, Long R (2013) Multidrug and extensively drug-resistant tuberculosis in Canada 1997–2008: demographic and disease characteristics. PLoS One 8(1): e53466

Miotto P, Bigoni S, Migliori GB, Matteelli A, Cirillo DM (2012) Early tuberculosis treatment monitoring by Xpert® MTB/RIF: Figure 1. Eur Respir J 39:1269–1271

Morgan M, Kalantri S, Flores L, Pai M (2005) A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. BMC Infect Dis 5:62

Mori T (2009) Usefulness of interferon-gamma release assays for diagnosing TB infection and problems with these assays. J Infect Chemother 15:143–155

Mortimer TD, Weber AM, Pepperell CS (2018) Signatures of selection at drug resistance loci in *Mycobacterium tuberculosis*. mSystems 3:e00108–e00117

Nathavitharana RR, Shi CX, Chindelevitch L, Calderon R, Zhang Z, Galea JT, Contreras C, Yataco R, Lecca L et al (2017) Polyclonal pulmonary tuberculosis infections and risk for multidrug resistance, Lima, Peru. Emerg Infect Dis 23:1887–1890

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382–388

Ninan MM, Gowri M, Christopher DJ, Rupali P, Michael JS (2016) The diagnostic utility of line probe assays for multidrug-resistant tuberculosis. Pathog Glob Health 110:194–199

Palomino JC, Martin A (2014) Drug Resistance Mechanisms in Mycobacterium tuberculosis. Antibiotics (Basel) 3(3):317–340

Pankhurst LJ, Elias CDO, Votintseva AA, Walker TM, Cole K, Davies J, Fermont JM, Gascoyne-Binzi DM, Kohl TA et al (2016) Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. Lancet Respir Med 4:49–58

Parsons LM, Somoskovi A, Urbanczik R, Salfinger M (2004) Laboratory diagnostic aspects of drug resistant tuberculosis. Front Biosci 9:2086–2105

Patel S, Paulsen C, Heffernan C, Saunders D, Sharma M, King M, Long R (2017) Tuberculosis transmission in the Indigenous peoples of the Canadian prairies. PLoS One 12(11):e0188189

Pepperell CS, Casto AM, Kitchen A, Granka JM, Cornejo OE, Holmes EC, Birren B, Galagan J, Feldman MW (2013) The role of selection in shaping diversity of natural *M. tuberculosis* populations. PLoS Pathog 9:e1003543

Public Health Agency of Canada (2014) Canadian tuberculosis standards, 7th edn, Ottawa

Public Health Agency of Canada (2019) Government of Canada. Government of Canada, Canada

Rachow, A., Zumla, A., Heinrich, N., Rojas-Ponce, G., Mtafya, B., Reither, K., Ntinginya, E. N., Ogrady, J., Huggett, J. and other authors. 2011. Rapid and accurate detection of *Mycobacterium tuberculosis* in sputum samples by Cepheid Xpert MTB/RIF assay—a clinical validation study. PLoS One 6**,** e20458

Rodwell TC (2019) Implementing WGS and culture-free NGS: an overview of challenges and solutions. In: Next-generation sequencing for drug resistant TB: Webinar Series. https://www.who.int/tb/treatment/Rodwell_NGS_WebinarSession1_Final.pdf?ua=1. Accessed 1 June 2020

Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, Wirth T, Jaenicke S, Schuback S et al (2013) Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. PLoS Med 10:e1001387

Sarmiento OL, Weigle KA, Alexander J, Weber DJ, Miller WC (2003) Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. J Clin Microbiol 41: 3233–3240

Schleusener V, Köser CU, Beckert P, Niemann S, Feuerriegel S (2017) *Mycobacterium tuberculosis* resistance prediction and lineage classification from genome sequencing: comparison of automated analysis tools. Sci Rep 7:46327

Schmidt K, Mwaigwisya S, Crossman LC, Doumith M, Munroe D, Pires C, Khan AM, Woodford N, Saunders NJ et al (2016) Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. J Antimicrob Chemother 72:104–114

Schürch AC, Schaik WV (2017) Challenges and opportunities for whole-genome sequencing-based surveillance of antibiotic resistance. Ann N Y Acad Sci 1388:108–120

Sester M, Sotgiu G, Lange C et al (2011) Interferon-{gamma} release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J 37(1):100–111

Sethi S, Yadav R, Mewara A, Dhatwalia SK, Sharma M, Gupta D (2012) Evaluation of in-house mpt64 real-time PCR for rapid detection of *Mycobacterium tuberculosis* in pulmonary and extra-pulmonary specimens. Braz J Infect Dis 16:493–494

Sharma MK (2011) The Purple Paper (National Collaborating Centre for Infectious Diseases), Issue No. 24, February 2011, Mycobacterium tuberculosis surveillance in Canada

Sharma M, Thibert L, Chedore P, Shandro C, Jamieson F, Tyrrell G, Christianson S, Soualhine H, Wolfe J (2011) A Canadian multicentre laboratory study for standardized second-line antimicrobial susceptibility testing of Mycobacterium tuberculosis. J Clin Microbiol 49:4112–4116

Somoskovi A, Parsons LM, Salfinger M (2001) The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in Mycobacterium tuberculosis. Respir Res 2:164–168

Steingart KR, Sohn H, Schiller I, Kloda LA, Boehme CC, Pai M, Dendukuri N (2013) Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev 2014(1):CD009593

Tamma PD, Fan Y, Bergman Y, Pertea G, Kazmi AQ, Lewis S, Carroll KC, Schatz MC, Timp W, Simner PJ (2018) applying rapid whole-genome sequencing to predict phenotypic antimicrobial susceptibility testing results among carbapenem-resistant *Klebsiella pneumoniae* clinical isolates. Antimicrob Agents Chemother 63:e01923–e01918

Thorne N, Borrell S, Evans J, Magee J, Viedma DGD, Bishop C, Gonzalez-Martin J, Gharbia S, Arnold C (2011) IS*6110*-based global phylogeny of *Mycobacterium tuberculosis*. Infect Genet Evol 11:132–138

Tyler AD, Christianson S, Knox NC, Mabon P, Wolfe J, Van Domselaar G, Graham M, Sharma MK (2016) Comparison of sample preparation methods used for the next-generation sequencing of Mycobacterium tuberculosis. PLoS One 11(2):e0148676

Tyler AD, Randell E, Baikie M, Antonation K, Janella D, Christianson S, Tyrrell G, Graham M, Van Domselaar G, Sharma MK (2017) Application of whole genome sequence analysis to the study of Mycobacterium tuberculosis in Nunavut, Canada. PLoS One 12(10):e0185656

U.S. National Library of Medicine (2020) Tuberculosis, advanced—chest x-rays [online]. https://medlineplus.gov/ency/imagepages/1607.htm. Accessed 29 June 2020

Votintseva AA, Bradley P, Pankhurst E, Del Ojo Elias C, Loose M, Nilgiriwala K, Chatterjee A, Smith EG, Sanderson N, Walker TM, Morgan MR, Wyllie DH, Walker AS, Peto TEA, Crook DW, Iqbal Z (2017) Same-day diagnostic and surveillance data for tuberculosis via whole-genome sequencing of direct respiratory samples. J Clin Microbiol 55(5):1285–1298

Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ et al (2013) Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. Lancet Infect Dis 13(2):137–146

Walker TM, Kohl TA, Omar SV, Hedge J, Elias CDO, Bradley P, Iqbal Z, Feuerriegel S, Niehaus KE et al (2015) Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study. Lancet Infect Dis 15:1193–1202

Wallis RS, Pai M, Menzies D, Doherty TM, Walzl G, Perkins MD, Lancet AZ (2010) Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet 375(9729):1920–37. https://doi.org/10.1016/S0140-6736(10)60359-5

Wang J-Y, Hsu H-L, Yu M-C, Chiang C-Y, Yu F-L, Yu C-J, Lee L-N, Yang P-C (2011) Mixed infection with Beijing and non-Beijing strains in pulmonary tuberculosis in Taiwan: prevalence, risk factors, and dominant strain. Clin Microbiol Infect 17:1239–1245

Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Pittius NCGV, Helden PDV (2004) Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med 169:610–614

Witney AA, Cosgrove CA, Arnold A, Hinds J, Stoker NG, Butcher PD (2016) Clinical use of whole genome sequencing for *Mycobacterium tuberculosis*. BMC Med 14:46

Woods GL, Brown-Elliott BA, Conville PS, Desmond EP, Hall GS, Lin G, Pfyffer GE, Ridderhof JC, Siddiqi SH et al (2011) Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes: approved standard. Clinical and Laboratory Standards Institute, Wayne

World Health Organization (2008) WHO Policy Statement [online]. https://www.who.int/tb/features_archive/policy_statement.pdf. Accessed 1 June 2020

World Health Organization (2018) Technical report on critical concentration for drug susceptibility testing of medicines used in the treatment of drug resistant tuberculosis. Geneva: (WHO/CDS/TB/2018.5). Licence CC BY-NC-SA 3.0 IGO. Major data contributors (K Andries, A Aubry, I Bastian, E Böttger, E Cambau, D Cirillo, P Claxton, E Desmond, J de Steenwinkel, K Dheda, A

Diacon, D Dolinger, A Engström, M Farhat, L Fattorini, S Heysell, D Hillemann, H Hoffmann, E Houpt, P Hsueh, Y Hu, N Ismail, J Jani, K Kaniga, P Keller, I Laurenson, J Limberis, G Lin, Y Liu, A López-Gavín, H Mauch, S Omar, M Palaci, S Peacock,T Prammananan, J Posey, L Rigouts, J Robledo, C Rodrigues, T Schön, M Sharma, T Shinnick, F Sirgel, S Somasundaram, T Sterling, J van Ingen, D van Soolingen, A Venter, N Veziris, C Villellas, R Warren, J Werngren, C Yang, W W Yew and D Zimenkov)

World Health Organization (2020) Global tuberculosis report 2020. World Health Organization, Geneva. Licence: CC BY-NC-SA 3.0 IGO. https://creativecommons.org/licenses/by-nc-sa/3.0/igo

Zakham F, Lahlou O, Akrim M, Bouklata N, Jaouhari S, Sadki K, Seghrouchni F, Elmzibri M, Benjouad A, other authors. (2012) Comparison Of A DNA based PCR approach with conventional methods for the detection of *Mycobacterium Tuberculosis* in Morocco. Mediterr J Hematol Infect Dis 4:e2012049

Zhang Y, Heym B, Allen B, Young D, Cole S (1992) 1992. The catalase—peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 358(6387):591–593

Zignol M, Cabibbe AM, Dean AS, Glaziou P, Alikhanova N, Ama C, Andres S, Barbova A, Borbe-Reyes A et al (2018) Genetic sequencing for surveillance of drug resistance in tuberculosis in highly endemic countries: a multi-country population-based surveillance study. Lancet Infect Dis 18:675–683

Zwolska Z (2005) Modern microbiological diagnostic methods of tuberculosis in clinical practice. Ukrainian Pulmonol J 3:70–71

Gut Microbes in Cardiovascular Diseases

11

K. K. Talwar, Mohit M. Bhagwati, and Amitabh Yaduvanshi

Abstract

Cardiovascular disease (CVD) has been one of the leading causes of morbidity and mortality in both developing and developed world. Lately, importance of diet and gut microbiota in the pathogenesis and management of CVD has been studied in animal and human models. Gut microbes not only regulate the metabolic pathways in the host but are also essential in homeostasis, and change in the composition of the gut microbiota (dysbiosis) has been associated with the development of CVD including atherosclerosis, hypertension, heart failure, diabetes, and obesity. Trimethylamine-*N*-oxide (TMAO), short-chain fatty acids (SCFA), secondary bile acids, and lipopolysaccharide/endotoxin (LPS) are among the important regulators of cardiovascular homeostasis, derived from gut microbes. This review discusses the normal gut microbiota, role of dysbiosis in cardiovascular disease pathogenesis, and potential therapeutic option for CVD by modulating gut microbiome including that of prebiotics, probiotics, and fecal microbiota transplantation (FMT).

Keywords

Cardiovascular diseases · Gut microbiota · Dysbiosis · TMAO · SCFA · Prebiotics · Probiotics

K. K. Talwar (✉)
Cardiac Sciences, PSRI Heart Institute, New Delhi, India

M. M. Bhagwati
Cardiology, PSRI Heart Institute, New Delhi, India

A. Yaduvanshi
Invasive Cardiology and Electrophysiology, PSRI Heart Institute, New Delhi, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_11

11.1 Introduction

Cardiovascular disease (CVD) is an ongoing epidemic with rising prevalence with social and economic development and is a major cause of morbidity and mortality in developing and developed countries. According to World Health Organization (WHO), 17.9 million people die every year due to CVD. This is about 31% of deaths worldwide. Apart from being a health issue, CVDs place a heavy burden on the economies of low- and middle-income countries (Cardiovascular Diseases (CVDs) 2017). Despite progress in medical therapy, it has been seen that only one in four CVD events can be prevented by medication. Therefore, there is an unmet demand regarding the prevention and treatment of CVD and more needs to be done. CVDs are usually associated with risk factors like hypertension, Type 2 diabetes mellitus (T2DM), atherosclerosis, and obesity. Among the modifiable risk factors for CVD, nutrition and dietary practices are key components. Hippocrates famously said, "All diseases begin in the Gut". This dictum holds true for CVD as well, and there is now increasing evidence that gut microbes play an important role in development of CVD. A heart–gut axis has been explained, where there is an effect of gut microbiota and microbiota-derived molecules on heart and vice versa. Gut microbiota is recognized as a regulator of metabolic pathways in the host and essential in homeostasis management, whereas a change in the composition of the gut microbiota (dysbiosis) has been associated with pathophysiological traits like atherosclerosis, hypertension, heart failure, arrhythmia, cardiac tumors, and others (Ahmad et al. 2019).

There is now an increased understanding of the role of gut bacteria in CVD through their metabolites, mainly (1) Formation of trimethylamine-*N*-oxide (TMAO), (2) Production of short-chain fatty acids (SCFA) (3) Regulator in intestinal bile acid metabolism, (4) Lipopolysaccharide/endotoxin (LPS) production-tissue inflammation. Gut microbiota and its relation to obesity is also an area of active interest among researchers. There are clinical reports demonstrating the disease-specific gut microbiome in patients with CVD, as well as the role of probiotics and prebiotics in modifying the microbiome to treat CVD. This chapter discusses the normal gut microbiota, role of dysbiosis in CVD pathogenesis, and the potential therapeutic option for CVD by modulating gut microbiome.

11.2 Gut Microbiota and Its Role in Homeostasis

The human microbiome project states that there are about 100 trillion microscopic life forms living on human body. The number is about 10 times more than all the cells in human beings, gut microbiota consists of bacteria, viruses, fungi, and viruses. Specific microbial profiles are associated with different organ systems in the body with highest microbe density present in colon. There are five major families in the intestinal flora: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia (Eckburg et al. 2005). The distribution of bacteria among different sites in gastrointestinal (GI) tract is constant with highest amount of that in ascending

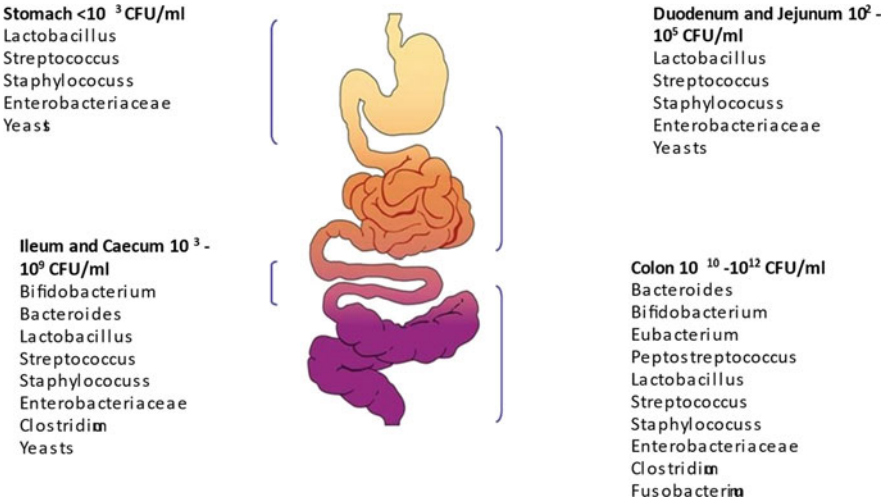


Fig. 11.1 Distribution of microbiota in GI tract

Table 11.1 Causes of dysbiosis

Unbalanced diet (high simple carbohydrate)
Diabetes mellitus, cancer, disease of liver and pancreas
Inflammatory process in intestine
Helminthic infestation in intestine
Uncontrolled rectal cleansing with enemas
Chronic and acute infections (HIV, HCV. Hep B, etc.)
Treatment with antibiotics
Chemotherapy, antiviral drugs, radiotherapy, hormone therapy

colon (Fig. 11.1). The phyla Bacteroidetes and Firmicutes compose of almost 90% of the gut microbiome (Gill et al. 2006). Gut microbiome in normal healthy human beings corresponds to a healthy bacteria balance between abundance of commensals and low proportions of general pathogens. A healthy bacteria balance means good bacteria overpower bad bacteria. Gut dysbiosis (Imbalance in gut microbiota) can lead to long-term susceptibility to disease. The ratio of the two most abundant microbes, Firmicutes to Bacteroidetes (F/B) varies between individuals and elevated F/B has been suggested to play a role in pathogenesis of various CVD like obesity and diabetes (Koliada et al. 2017; Pascale et al. 2019). The causes of dysbiosis are mentioned in Table 11.1.

11.3 Gut Dysbiosis and CVD

SCFA are generated by colonic bacteria from the fermentation of complex carbohydrates of dietary fiber. SCFA includes acetate, propionate, and butyrate. The ratio of propionate: butyrate: acetate is 1:1:3 in the GI tract (Tazoe et al. 2008).

Propionate is predominantly produced by Bacteroidetes, whereas butyrate is predominantly produced by Firmicutes. SCFAs perform several roles in normal homeostasis. Propionate and acetate serve as substrates for gluconeogenesis and lipogenesis. Butyrate acts as main energy source for colonic epithelial cells and improves insulin sensitivity. SCFAs function as signaling molecules, activating G-protein coupled receptors (GPR) GPR41 and GPR43 which are expressed in the intestinal epitheliums well as in hepatic and muscle cells, which helps in secretion of glucagon-like peptide-1 (GLP-1), peptide YY (PYY) (Zhou et al. 2020; Samuel et al. 2008). These molecules play an important role in host energy homeostasis. GLP-1 enhances the secretion of insulin and PYY is an anorexigenic (reduces appetite). SCFA can thus beneficially modulate adipose tissue, skeletal muscle and liver tissue function. It can contribute to improved glucose homeostasis and insulin sensitivity and has a potential role as metabolic targets to prevent and counteract obesity and its associated disorders in glucose metabolism and insulin resistance. In patients with T2DM, GLP-1 receptor agonists not only affect improvements in impaired beta cell and alpha cell function, suppress appetite, and induce weight loss but also possess multiple cardiovascular protective properties that have a beneficial impact on atherosclerotic cardiovascular disease (ASCVD) morbidity and mortality. Drugs like Liraglutide is now also approved for use in heart failure for its cardioprotective effects (Marso et al. 2016). SCFA depletion can also lead to Heart failure (HF) due to disruption of intestinal mucosal barrier and translocation of endotoxins into blood stream (Zhou et al. 2020).

Trimethylamine-*N*-oxide (TMAO) is yet another molecule which has been shown to play a role in pathogenesis of CVD. Dietary choline, phosphatidylcholine, carnitine which are found in abundance in red meat, milk, and eggs are metabolized by microbial enzymes and trimethylamine (TMA) is produced in intestine (Li et al. 2017). TMA enters liver via portal circulation and undergoes oxidation by flavin monooxygenases (FMOs), especially FMO3 to produce TMAO (Bennett et al. 2013). Increased TMAO levels show a strong positive correlation with atherosclerosis (Wang et al. 2011). TMAO induces inflammation by stimulating the release of inflammatory markers like IL-18 and IL-1β, resulting in the recruitment of leucocytes and endothelial cells and thus accentuating atherosclerosis. This action is done via activating nuclear factor kappa beta (NFK-β) pathway (Seldin et al. 2016). Apart from atherosclerosis, TMAO is also responsible for prothrombotic state by platelet activation resulting in acute myocardial infarction (MI) and stroke, extracellular matrix (ECM) production and myocardial fibrosis leading to heart failure (HF). TMAO is also associated with glucose intolerance and diabetes mellitus (Zhou et al. 2020). TMAO/TMA-producing bacteria belong mainly to the families of Deferribacteraceae, Anaeroplasmataceae, Prevotellaceae, and Enterobacteriaceae. Also, the bacteria representing Firmicutes and Proteobacteria phyla produce TMA/TMAO. Tang et al. showed increased levels of TMAO in plasma and urine after phosphatidylcholine or carnitine challenge (two hard-boiled eggs and deuterium-labeled phosphatidylcholine). These levels decreased on antibiotic therapy and reappeared on withdrawing antibiotic therapy (Tang et al. 2013). In another study, it was shown that with increasing TMAO levels, major adverse cardiovascular

outcomes (MACE) increase. This association of TMAO with CVD clearly highlights the role of gut microbiota in CVD. On the contrary, it has been identified that some methanogenic bacteria belonging to the order Methanobacteriales utilize only methyl compounds including TMA as substrate. These bacteria are natural inhabitants of the gut as well. Also, various bacteria grow anaerobically using TMAO as an alternative terminal electron acceptor of a respiratory transport chain. TMAO is reduced to volatile TMA during this reaction. Therefore, the concept of colonizing human gut with such bacteria to reduce TMAO levels can be further explored. It has been shown that a structural analog of choline, 3,3-dimethyl-1-butanol (DMB) when used to inhibit microbial enzyme TMA lyase, suppressed TMAO levels and atherosclerosis in animal models (Chen et al. 2017). Therefore, this alternative approach of targeting bacterial enzymes responsible for TMAO formation rather than bacteria per se, can be a potential therapy.

Plasma cholesterol is a well-established risk factor for CVD. It is a precursor of Vitamin D, steroids, and bile acids (BA). Primary BA are derived from cholesterol in the liver, are then conjugated to either glycine or taurine, and then excreted into the small intestine to aid lipid emulsification. BA transporters, which are present in terminal ileum reabsorb almost 95% of BAs, which then undergo recycling in the liver, to be secreted again. Primary BA which has escaped reabsorption gets converted to secondary bile acids (SBA) by colonic gut bacteria. SBA are less effective in emulsifying fat for absorption (Lau et al. 2017). Gut microbiota can catalyze the deconjugation of primary BA to form SBA through bacterial bile-salt hydrolase (BSH) activity. BSH is found in Clostridium, Bifidobacterium, Lactobacillus, etc. A small amount of microbe-derived SBA act as hormones after entering circulation. They affect signaling pathways involved in metabolism, energy expenditure, and inflammation. Since SBA are less soluble, they are less likely to be reabsorbed and more likely to be excreted, providing a pathway for cholesterol elimination. This intricate cycle constitutes the enterohepatic circulation of BAs, governed by BA-signaling of the hepatic farnesoid X receptor (FXR). SBAs cause activation of macrophages and production of cytokines via G protein-coupled BA receptor (TGR5). BA are an important pathway for cholesterol elimination from the body via feces and decreasing plaque burden (Ridlon et al. 2014). Low levels of SBA show anti-inflammatory effects, whereas higher concentration of SBAs is harmful. So, another way in which the gut microbiota influences its host is by acting as a regulator of bile acid metabolism. Lipopolyscahrides and peptodogylcans play a role in inflammation, atherosclerosis insulin resistance, and obesity via Toll-like receptors (TLRs), NOD-like receptors (NLRs) (Tang et al. 2017). The role of gut microbes in CVDs is summarized in Fig. 11.2.

Obesity is a growing epidemic worldwide. It has been found in mice and human studies that obesity is associated with higher Firmicutes to Bacteroidetes (F/B) ratio. The Bacteroidetes increase with weight loss (Indiani et al. 2018; Ley et al. 2005). In monozygotic twins, obesity is associated with decreased diversity of microbiome, decreased Bacteroidetes, and increased genes for carbohydrate and fat metabolism (Davis 2016). It has been shown that antibiotic use in infancy leads to increased obesity in later life and perinatal decreased exposure to L. rhamnosus cause weight

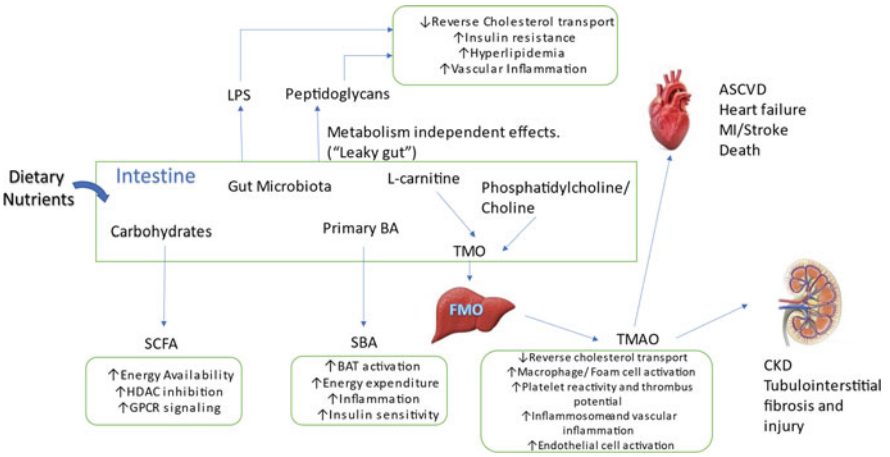


Fig. 11.2 Role of gut microbes in CVD

gain in childhood (Angelakis and Raoult 2018). The impact of microbiota on obesity is significant. It has been seen that individuals with a low bacterial richness are characterized by more marked overall adiposity, insulin resistance and dyslipidemia, and a more pronounced inflammatory phenotype when compared with high bacterial richness. As discussed before, SCFA produced by gut microbes stimulate GLP-1 secretion. The clinical impact of GLP-1 receptor agonist was recently demonstrated in STEP-1 study where once weekly Semaglutide, a GLP-1 analogue caused significant weight loss in obese individuals (Wilding et al. 2021). Clinical implications of role of gut microbiome in various CVD has been summarized in Table 11.2.

11.4 Therapeutic Interventions to Target Dysbiosis and CVD

In the last few years, many attempts have been made to target gut microbiota as a therapeutic target to improve outcomes in CVD. Gut microbiota can be altered favorably by (1) Dietary intervention, (2) bacteriotherapy: prebiotic, probiotics, and synbiotics, and (3) Fecal microbiota transplantation (FMT).

11.5 Dietary Intervention

Dietary intervention has been the oldest and easiest method of altering gut microbiota. Dietary habits influence composition of microbiota and digestion/absorption of nutrients. Dietary components like macronutrients, fiber, polyphenols, prebiotics, and probiotics alter gut microbiome and production of metabolites of microbiome like SCFA. It has been shown that intake of animal protein, amino acids, and saturated fats is associated with increase in Bacteroides enterotype in the gut

Table 11.2 Clinical implications of gut dysbiosis

2012 Nat. Commun.	12 patients with symptomatic atherosclerosis (myocardial infarction or cerebrovascular events) and 13 age- and sex-matched healthy individuals	Sweden	atherosclerosis	*Collinsella*	*Eubacterium* *Roseburia*
2015 J. Am. Heart Assoc.	141 patients with stroke and transient ischemic attack (stroke/TIA patients) and 94 asymptomatic controls	China	Stroke/TIA patients	*Enterobacteriaceae* *Proteobacteria* *Escherichia/Shigella*	*Bacteroidetes* *Bacteroidales* *Bacteroidaceae* *Bacteroides*
2016 J. Atheroscler. Thromb.	39 coronary artery disease (CAD) patients, 30 age- and sex-matched no-CAD controls with coronary risk factors, and 50 healthy volunteers without coronary risk factors	Japan	CAD	*Firmicutes/ Bacteroidetes ratio* *Lactobacillales*	*Bacteroides* + *Prevotella*
2017 Nat. Commun	218 individuals with atherosclerotic cardiovascular disease (ACVD) and 187 healthy controls	China	ACVD	*Enterobacteriaceae* *E. coli* *Klebsiella* spp. *Enterobacter aerogenes* *Streptococcus* spp. *Lactobacillus salivarius* *Solobacterium moorei* *Atopobium parvulum* *Ruminococcus gnavus* *Eggerthella lenta*	*Roseburia intestinalis* *Faecalibacterium* cf. *Prausnitzii* *Bacteroides* spp. *Prevotella copri* *Alistipes shahii*

microbes and decrease in the Prevotella enterotype. Vegetarians are found to have rich Prevotella enterotype. In one more study, it was seen that European children having western diet (High protein and Fat) have predominant Bacteroides enterotype and Burkina Faso children consuming typical high-carbohydrate and low-animal protein diet had Prevotella enterotype microbiome (Wu et al. 2011; De Filippo et al. 2010). Lately, Mediterranean diet has been found to be protective for CVD. This diet is rich in fruits, vegetables, grains, and legumes and low in processed carbohydrates and red meat. This diet reduces inflammation and oxidative stress, increases nitric oxide availability, and modulation of gut microbiota to improve cardiovascular function (Eckel et al. 2014). Whereas a diet high in saturated fats decrease gut microbe diversity and commensals like Bifidobacterium and is harmful. Diet rich in polyphenols, an aromatic compound found mainly in plant-based beverages and foods, such as apples, berries, citrus, cocoa, tea, and coffee are found to have antidiabetic and cardioprotective properties. These polyphenols get metabolized in the gut by microbes and gut microbiota influence their metabolites. Polyphenols like Anthocyanins and Flavonoids are shown to modulate F/B ratio and thus have metabolic effects. Anthocyanins also have antiplatelet effect beneficial in atherosclerosis, and nitric oxide formation in blood vessels causing vasodilation (Mayta-Apaza et al. 2018; Gaiz et al. 2018). Grape wine and Red wine containing polyphenols also have been found to have cardioprotective effects. One more polyphenol, Quercetin which is a member of the flavonoid family is found to increase F/B ratio, increase Bacteroides vulgatus and Akkermansia, and reduce Eubacterium cylindroides and Bilophilia wadsworthia (Ahmad et al. 2019). All of this is found to have beneficial effects in management of obesity.

11.6 Bacteriotherapy: Prebiotics, Probiotics and Synbiotics

Prebiotics are non-digestible compounds commonly derived from fiber-rich food and found to affect gut microbiome in a favorable manner. It includes mainly Inulin, Oligosaccharide, Oligofructose, Fructooligosaccharide. A high fiber-rich diet modifies the gut microbiota to augment bacteria-producing acetate. Acetate modifies outcomes favorably in CVD by its action on inflammation and hypertrophy through transcription factor Egr1 (Marques et al. 2017). Inulin is made up of fructose polymers found in chicory root, wheat, onion, banana, and garlic which are fermented in colon to SCFA. Oligofructose enriched inulin was found to increase Bifidobacterium in the gut and resulted in weight loss. Prebiotics are also shown to increase levels of butyrate-producing bacteria (Feacalibacterium prausnitzii) and Mucin degrading bacteria (Akkermansia muciniphila). β-glucan is a prebiotic which was found to have favorable effects on CVD by decreasing total cholesterol and low-density lipoprotein (LDL) (Ahmad et al. 2019). Probiotics are live bacteria which promote favorable gut microbiota composition. Probiotics modulate pH, stimulate immunity, prevent inflammation, improve gut metabolism, preventing bacterial enzyme activity, and production of ammonia on one hand, and stimulating digestive enzymes on another hand. The most studied bacteria as probiotics belong

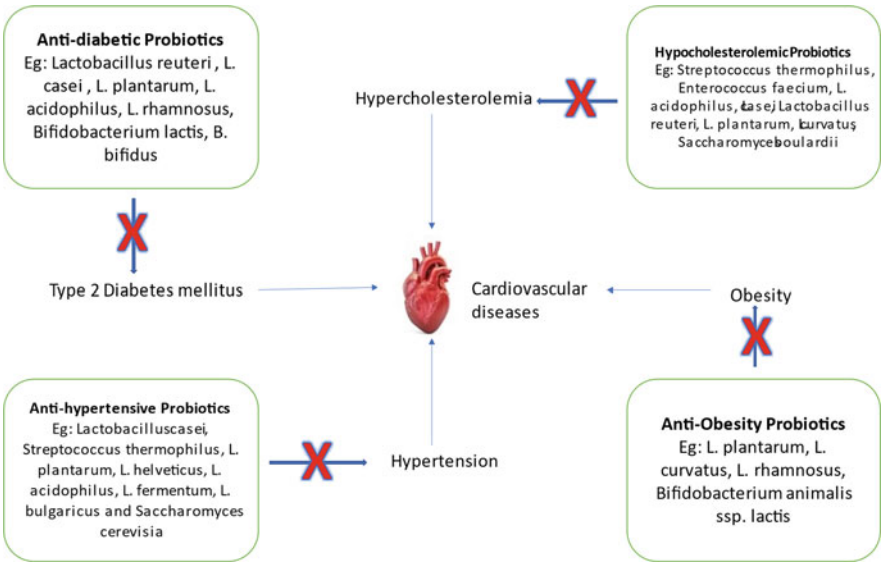


Fig. 11.3 Probiotics in CVD

to Bifidobacteria, Lactobacilli, Lactococci, and Streptococci group (Ahmad et al. 2019). These probiotics when administered in various combinations and strength are found to have favorable effects in lipid profile (decreasing LDL and increasing high-density Lipoprotein), weight loss and decrease in blood pressure. Effect of various probiotics on CVD is summarized in Fig. 11.3. The combination of probiotics and prebiotics is known as synbiotics. Synbiotics help in promoting growth and survival of favorable substrate for fermentation and good bacteria in the gut and thus promoting beneficial microbial ecosystem. Synbiotic intervention has been found to decrease weight and Metabolic syndrome in mice with high fat diet (Ke et al. 2019).

11.7 Fecal Microbiota Transplantation (FMT)

FMT is a process involving transfer of fecal sample (own or others') in order to restore gut microbiome dysbiosis and function. Fecal sample constitutes over 70% of gut microbiome. It's a proven therapy in Clostridium difficle infection and Ulcerative colitis. It has been mooted as a prospective therapy in metabolic syndrome. One study involving transplantation of gut bacteria from lean persons to obese individuals was found to increase insulin sensitivity in obese individuals. The gut microbiome of obese individuals had lower diversity high levels of Bacteroidetes and reduced Clostridium levels, which upon allogenic gut microbiota transplant changed to a highly diverse microbiome with 2.5 times increase in butyrate-producing bacteria Roseburia intestinalis (Vrieze et al. 2012). The impact of FMT

has also been shown in mice where hypertension was transferrable when FMT was done from hypertensive mice to normotensive mice. FMT is still an evolving science and proper studies are required to lay down protocols for donor and recipient selection, mode of transplant (Upper vs lower gastrointestinal tract). FMT also carries a small risk of endotoxin transfer or infection transfer causing complications including death (Kelly et al. 2015).

As already discussed, 3,3-dimethyl-1-butanol (DMB) when used to inhibit microbial enzyme TMA lyase, suppresses TMAO levels. TMA and TMAO levels are linked to many CVD including atherosclerosis and thus this understood pathway can be used as therapeutic target to improve outcomes. DMB is a potent and irreversible inhibitor and it does not affect commensals. One more potential advantage of DMB is that unlike antiplatelet agents, it does not affect platelet function and does not carry risk of bleeding.

11.8 Conclusion

Enough evidence is available to establish that gut microbiota is essential in homeostasis, with some protective role against many diseases including CVD. Dysbiosis, in many ways, play a role in the pathogenesis of CVDs via various metabolic pathways, such as inducer of endotoxemia, formation of TMAO, production of SCFA, and as a regulator in intestinal bile acid metabolism. There are lifestyle management and potential therapies targeting dysbiosis, including dietary intervention, pre/probiotics, TMAO inhibitors, and fecal microbiota transplantation (FMT) which present new opportunities for CVD management. Our understanding of the influence of gut microbiota on cardiometabolic health is evolving with time and various innovative therapeutic options are still in the developing phase. Further studies, focusing on a more specific and mechanistic understanding of the gut microbiota in the pathogenesis of CVD, are necessary to develop novel diagnostic and therapeutic strategies for CVD using this route of intervention.

References

Ahmad AF, Dwivedi G, O'Gara F, Caparros-Martin J, Ward NC (2019) The gut microbiome and cardiovascular disease: current knowledge and clinical potential. Am J Physiol Heart Circ Physiol 317(5):H923–H938

Angelakis E, Raoult D (2018) Gut microbiota modifications and weight gain in early life. Hum Microbiome J 7–8:10–14

Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J et al (2013) Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell Metab 17(1):49–60

Cardiovascular Diseases (CVDs) (2017, May 17) World Health Organisation. https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds). Accessed online

Chen K, Zheng X, Feng M, Li D, Zhang H (2017) Gut microbiota-dependent metabolite trimethylamine N-oxide contributes to cardiac dysfunction in Western diet-induced obese mice. Front Physiol 8:139

Davis CD (2016) The gut microbiome and its role in obesity. Nutr Today 51(4):167–174
De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107:14691–14696
Eckburg PB, Bik EM, Bernstein CN et al (2005) Diversity of the human intestinal microbial flora. Science 308(5728):1635–1638
Eckel RH, Jakicic JM, Ard JD, de Jesus JM, Houston Miller N, Hubbard VS et al (2014) American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2013 AHA/ACC guideline on lifestyle management to reduce cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 63:2960–2984. [Erratum in: J Am Coll Cardiol (2014) 63: 3027–3028]
Gaiz AA, Mosawy S, Colson N, Singh I (2018) Potential of anthocyanin to prevent cardiovascular disease in diabetes. Altern Ther Health Med 24:40–47
Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312(5778):1355–1359
Indiani CMDSP, Rizzardi KF, Castelo PM, Ferraz LFC, Darrieux M, Parisotto TM (2018) Childhood obesity and Firmicutes/Bacteroidetes ratio in the gut microbiota: a systematic review. Child Obes 14(8):501–509
Ke X, Walker A, Haange SB, Lagkouvardos I, Liu Y, Schmitt-Kopplin P et al (2019) Synbiotic-driven improvement of metabolic disturbances is associated with changes in the gut microbiome in diet-induced obese mice. Mol Metab 22:96–109
Kelly CR, Kahn S, Kashyap P, Laine L, Rubin D, Atreja A et al (2015) Update on fecal microbiota transplantation 2015: indications, methodologies, mechanisms, and outlook. Gastroenterology 149:223–237
Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V et al (2017) Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC Microbiol 17(1):120
Lau K, Srivatsav V, Rizwan A, Nashed A, Liu R, Shen R et al (2017) Bridging the gap between gut microbial dysbiosis and cardiovascular diseases. Nutrients 9:859–874
Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI (2005) Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 102:11070–11075
Li XS, Obeid S, Klingenberg R, Gencer B, Mach F, Räber L et al (2017) Gut microbiota-dependent trimethylamine N-oxide in acute coronary syndromes: a prognostic marker for incident cardiovascular events beyond traditional risk factors. Eur Heart J 38(11):814–824
Marques FZ, Nelson E, Chu PY, Horlock D, Fiedler A, Ziemann M et al (2017) High-fiber diet and acetate supplementation change the gut microbiota and prevent the development of hypertension and heart failure in hypertensive mice. Circulation 135:964–977
Marso SP, Daniels GH, Brown-Frandsen K, Kristensen P, Mann JF, Nauck MA et al (2016) LEADER Steering Committee; LEADER Trial Investigators. Liraglutide and cardiovascular outcomes in type 2 diabetes. N Engl J Med 375(4):311–322
Mayta-Apaza AC, Pottgen E, De Bodt J, Papp N, Marasini D, Howard L et al (2018) Impact of tart cherries polyphenols on the human gut microbiota and phenolic metabolites in vitro and in vivo. J Nutr Biochem 59:160–172
Pascale A, Marchesi N, Govoni S, Coppola A, Gazzaruso C (2019) The role of gut microbiota in obesity, diabetes mellitus, and effect of metformin: new insights into old diseases. Curr Opin Pharmacol 49:1–5
Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS (2014) Bile acids and the gut microbiome. Curr Opin Gastroenterol 30:332–338
Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK et al (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding g protein-coupled receptor, gpr41. Proc Natl Acad Sci U S A 105:16767–16772

Seldin MM, Meng Y, Qi H, Zhu W, Wang Z, Hazen SL et al (2016) Trimethylamine N-Oxide promotes vascular inflammation through signaling of mitogen-activated protein kinase and nuclear factor-κβ. J Am Heart Assoc 5(2):e002767

Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X et al (2013) Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 368(17):1575–1584

Tang WH, Kitai T, Hazen SL (2017) Gut microbiota in cardiovascular health and disease. Circ Res 120(7):1183–1196

Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki S-I, Kuwahara A (2008) Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol 59(Suppl 2): 251–262

Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF et al (2012) Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 143:913–9166.e7

Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B et al (2011) Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472(7341):57–63

Wilding JPH, Batterham RL, Calanna S, Davies M, Van Gaal LF, Lingvay I et al (2021) STEP 1 Study Group. Once-weekly semaglutide in adults with overweight or obesity. N Engl J Med 384(11):989

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA et al (2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105–108

Zhou W, Cheng Y, Zhu P, Nasser MI, Zhang X, Zhao M (2020) Implication of gut microbiota in cardiovascular diseases. Oxid Med Cell Longev 2020:5394096

Heart Failure: Future Perspectives

12

Ajay Bahl and Vibhuti Sharma

Abstract

Heart failure (HF) is a major public health problem. Indians are especially at risk since common underlying conditions like diabetes mellitus and coronary artery disease occur at a younger age. Heart failure preserved ejection fraction will be increasingly encountered as the population ages. Future approaches are likely to be more individualized. These will include better disease characterization with increasing use of genotyping and biomarkers. Technological advances in assist devices and total artificial hearts, and use of organ care systems for preservation of donor hearts are options for patients with end-stage heart failure.

Keywords

Heart failure · Epidemiological transition · Genetics · Artificial heart

12.1 Introduction

Heart failure (HF) is a clinical syndrome characterized by the inability of the heart to pump blood commensurate with the tissue requirements or do so only at raised filling pressures (Braunwald 1992). It is the end stage of a wide spectrum of cardiovascular diseases and is a major public health problem especially in societies with large elderly populations (McMurray and Stewart 2000). Like many developing countries, India too is in a unique situation. The HF problem has differences from that seen in the west. The country straddles several stages of the epidemiological transition (Omran 1971). Not only is India burdened with infectious disease outbreaks and

A. Bahl (✉) · V. Sharma
Department of Cardiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_12

malnutrition seen in more primitive societies but also faces the increasing prevalence of degenerative diseases seen in the west (Huffman and Prabhakaran 2010). In addition, there are economic and social disparities across different regions. Population-based data is also lacking in the Indian population.

To fully grasp the scope of the problem, it is important to clearly define HF. Pathophysiological definitions are of limited value when HF is to be diagnosed in any given individual. Diagnostic criteria like Framingham have their drawbacks and diagnosis of HF may be difficult in a clinical setting since the disease spectrum varies from asymptomatic individuals with heart disease and compromised cardiac function to those who are severely symptomatic and require hospitalization (Maestre et al. 2009). Abnormal haemodynamics may be precipitated only on exertion and not be apparent at rest. In addition, a large spectrum of conditions and risk factors responsible for HF also make epidemiological studies difficult.

HF is divided into three main groups as per the European Society of cardiology guidelines (Ponikowski et al. 2016). These are HF with preserved ejection fraction (HFpEF) if LVEF is $\geq$50%, HF with reduced EF (HFrEF) if LVEF is $<$40%. Patients in a grey zone with LVEF of 40–49% are labelled HF with mid-range ejection fraction (HFmrEF). American heart association guidelines are also similar (Yancy et al. 2013). However, they use the term 'HFpEF borderline' instead of HFmrEF for patients with LVEF 40–49%.

Advances in HF therapy over the past few decades have mainly been in the form of imaging, biomarkers, drug and device therapy, prevention of sudden death, and left ventricular assist devices. These have been validated in large, randomized trials. Future therapy may focus on individualized therapy based on the patient profile.

12.2 Indian Perspective

The spectrum of HF has evolved over time. The first large Indian study HF by Rustom Jal Vakil in 1949 found HF was common even at that time (Vakil 1949). His report on 1281 hospitalized HF patients found rheumatic heart disease (RHD) in 29.3% and syphilis in 11.7% patients (Vakil 1949). RHD is much less common and syphilitic heart disease has virtually disappeared. Although RHD has declined in percentage terms, it continues to be a significant public health problem. Coronary artery disease (CAD) and hypertension, the most common etiologies today accounted for less than one-third of cases in the mid-twentieth century. This reflects the rapid cardiovascular epidemiological transition in India over the past decades. Currently, CAD, hypertension and diabetes mellitus are the most important risk factors for future development of HF (Huffman and Prabhakaran 2010). Prevalence of all three conditions is increasing in both urban and rural areas (Kumar et al. 2006). Both CAD and diabetes mellitus occurs at a younger age as compared to the Caucasian population (Huffman and Prabhakaran 2010). This translates into increased HF prevalence at a younger age. Early age of onset may make HF a bigger public health problem in India as compared to the west. The INTERHEART study revealed that myocardial infarction risk in Indians was largely attributable to the nine

conventional risk factors (abnormal lipids, hypertension, diabetes mellitus, smoking, abdominal obesity, consumption of fruits, vegetables and alcohol, regular physical activity and psychosocial factors) (Yusuf et al. 2004). The risk factors were similar to those in other ethnic groups. This study dispelled the myth that non-conventional risk factors were mainly responsible for early onset of CAD in Indians. HFpEF is less frequently seen in the Indian population as compared to the west. This is possibly because of a younger population, since HFpEF is mainly a disease of the elderly. HFpEF defined as LVEF $\geq$45% constituted only 26% of the hospitalized HF patients in the Trivandrum HF registry as compared to over 50% in the contemporary western HF data (Harikrishnan et al. 2015). The Indian council of medical research-sponsored national HF registry is expected to provide comprehensive Indian data on acute HF by 2021 (Harikrishnan et al. 2019).

12.3 Future Approaches

HF will burden the health care system and consume significant health care resources in the future. The most effective way of reducing the HF burden will be targeting the common risk factors. These involve public health strategies to tackle the epidemic of CAD, hypertension, obesity and diabetes mellitus. HF strategies for management HFrEF and HFpEF differ. There has been immense progress in management of HFrEF over the past few decades. Large megatrials including thousands of thousands of patients have tested different therapies. These trials showed that drug therapies including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, aldosterone receptor antagonists and sodium-glucose cotransporter-2 inhibitors have vastly improved outcomes of patients with HFrEF (Domanski et al. 2003; McMurray et al. 2019; Ponikowski et al. 2016; Zannad et al. 2011). Devices including cardiac resynchronization therapy and implantable cardioverter defibrillators further enhance outcomes (Sethi and Dhall 2011).

Despite these enhancements, several patients develop end-stage disease with refractory HF. Option for these patients is limited to heart transplant, left ventricular assist devices or total artificial heart. Basic heart transplantation protocols haven't changed much. The introduction of sirolimus has made non-nephrotoxic immunosuppression possible. Availability of donor hearts is the major limiting factor since donation is usually restricted to brain-dead donors with preserved cardiac function. To increase the availability of donor hearts, donation after cardiac death may be expanded. Currently, retrieval after cardiac arrest is limited to only a few centres (Chew et al. 2019). This would require further developments in cost-effective organ care systems to manage the hearts retrieved from donors after cardiac arrest. Further developments in organ care systems have the potential to increase numbers of heart transplants globally.

Left ventricular assist devices and total artificial hearts have several potential advantages over heart transplantation. These are manufactured devices and thus not limited by donor availability. In addition, these are available off the shelf and can be carried out as elective procedures rather than as unplanned emergencies as in heart

transplant. Left ventricular assist devices may also be individualized depending on patient characteristics. Major advances have taken place in left ventricular assist device technology with miniaturization and improved-flow characteristics in newer devices (Goldstein et al. 2020; Schramm et al. 2020). These devices however support only one ventricle and thus total artificial heart becomes important in patients with associated right heart dysfunction. Left ventricular assist device and total artificial heart technologies are likely to a major thrust area in cardiology research. Battery life has been a major limiting factor. A drive line connected to an external power source is the Achilles heel of a left ventricular assist device and is a source of infection. Work on percutaneously chargeable, efficient, high-capacity batteries is important for future development of these devices. Further miniaturization and improved biocompatible surfaces are also an important area of research. It is possible that in a decade assist device results may rival or even better those of heart transplant.

Unfortunately, the HFpEF scenario is very different. Unlike HFrEF, no specific therapy has been shown to improve survival so far. Large megatrials using different groups of drugs have not shown survival benefits (Cleland et al. 2006; Massie et al. 2008; Solomon et al. 2019; Yusuf et al. 2003). Sodium glucose co-transport inhibitors have shown some promise, though results from large randomized trials are awaited (Anker et al. 2019; Kato et al. 2019). Loop diuretics cause symptomatic improvement but do not improve outcomes. This indicates that one-size-fits-all type of therapy may not work in HFpEF and individual phenotypes need to be identified. The approach to treatment of HFpEF should now shift away from large trials to the identification and treatment of specific phenotypes. There has been some progress in this area with the identification of transthyretin cardiac amyloidosis causing HFpEF in a small subset of patients (Driggin and Maurer 2020). Tafamidis, a transthyretin tetramer stabilizer improves survival and symptoms in these patients (O'Meara et al. 2020). HFpEF is associated with several comorbidities like hypertension, diabetes mellitus, obesity, chronic renal disease and atrial fibrillation. Further characterization of subsets and comorbidities each with differing treatment modalities would likely be the approach to managing HFpEF patients in future.

Another area of HF research that has been disappointing is cell replacement therapy. Large number of trials using stem cells in acute myocardial infarction and HF have been carried out. Most of these trials have simply injected various types of stem cells either in the coronary arteries or directly into the myocardium. Many of these have shown either no benefit or only a marginal benefit (Cerrone et al. 2008; Nair et al. 2015). Better understanding of stem cell biology and its interaction with myocardial tissue is needed.

Genetic contribution to HF is being increasingly recognized. In fact, cardiomyopathies commonly are familial disorders transmitted in a Mendelian inheritance pattern. These mutations are however spread over a large number of genes including sarcomeric, cytoskeletal, ion channel and energy pathway genes (Bondue et al. 2018; Christoforou and Gearhart 2007; Paldino et al. 2018). Next-generation sequencing has made it possible to rapidly identify these mutations, build a reliable and large database to characterize individual sequence variations. Most

sequence variations have been reported in a small number of patients. Many of these are non-pathogenic. Understanding the significance of tens of thousands of sequence variations is a huge task. Bioinformatics tools are useful but only predictive. Functional studies and good phenotypic characterization are needed but require resources. Much more complex is the role of sequence variations that only modify the phenotype and increase risk of HF. Unlike Mendelian mutations, these only contribute to the risk of HF. Understanding these variants requires large databases that can only be achieved by multicentre collaborations. Some of the common sequence variations like a 25 basepair deletion in intron 32 and p.Asp389Val in cardiac myosin binding protein C gene (*MyBPC3*) are found only in people of Indian descent (Dhandapany et al. 2009; Viswanathan et al. 2018). Characterization of sequence variations is a work that has only begun and is likely to continue over several decades. As our understanding of different pathways and their interactions increases, a systems biology approach may be used in the diagnosis and management of HF patients.

12.4 Conclusions

HF is one of the major public health problems of the twenty-first century. Major advances in HF management have involved large, randomized trials that apply to large groups of patients. An individualized, multi-pronged approach combining better disease characterization, risk factor control, genotyping, further developments in artificial heart technologies, cell replacement therapies and a systems biology approach are the future.

References

Anker SD, Butler J, Filippatos GS, Jamal W, Salsali A, Schnee J, Kimura K, Zeller C, George J, Brueckmann M, Zannad F, Packer M, EMPEROR-Preserved Trial Committees and Investigators (2019) Evaluation of the effects of sodium-glucose co-transporter 2 inhibition with empagliflozin on morbidity and mortality in patients with chronic heart failure and a preserved ejection fraction: rationale for and design of the EMPEROR-Preserved Trial. Eur J Heart Fail 21(10):1279–1287. https://doi.org/10.1002/ejhf.1596. Epub 2019 Sept 16

Bondue A, Arbustini E, Bianco A, Ciccarelli M, Dawson D, De Rosa M, Hamdani N, Hilfiker-Kleiner D, Meder B, Leite-Moreira AF, Thum T, Tocchetti CG, Varricchi G, Van der Velden J, Walsh R, Heymans S (2018) Complex roads from genotype to phenotype in dilated cardiomyopathy: scientific update from the Working Group of Myocardial Function of the European Society of Cardiology. Cardiovasc Res 114(10):1287–1303. https://doi.org/10.1093/cvr/cvy122

Braunwald E (1992) Heart disease: a textbook of cardiovascular medicine, 4th edn. Saunders, Philadelphia

Cerrone M, Remme CA, Tadros R, Bezzina CR, Delmar M (2008) Beyond the one gene-one disease paradigm: complex genetics and pleiotropy in inheritable cardiac disorders. Circulation 140(7):595–610. https://doi.org/10.1161/CIRCULATIONAHA.118.035954. Epub 2019 Aug 12

Chew HC, Iyer A, Connellan M, Scheuer S, Villanueva J, Gao L, Hicks M, Harkness M, Soto C, Dinale A, Nair P, Watson A, Granger E, Jansz P, Muthiah K, Jabbour A, Kotlyar E, Keogh A,

Hayward C, Graham R, Spratt P, Macdonald P, Dhital K (2019) Outcomes of donation after circulatory death heart transplantation in Australia. J Am Coll Cardiol 73(12):1447–1459. https://doi.org/10.1016/j.jacc.2018.12.067

Christoforou N, Gearhart JD (2007) Stem cells and their potential in cell-based cardiac therapies. Prog Cardiovasc Dis 49(6):396–413. https://doi.org/10.1016/j.pcad.2007.02.006

Cleland JG, Tendera M, Adamus J, Freemantle N, Polonski L, Taylor J, PEP-CHF Investigators (2006) The perindopril in elderly people with chronic heart failure (PEP-CHF) study. Eur Heart J 27(19):2338–2345. https://doi.org/10.1093/eurheartj/ehl250. Epub 2006 Sept 8

Dhandapany PS, Sadayappan S, Xue Y, Powell GT, Rani DS, Nallari P, Rai TS, Khullar M, Soares P, Bahl A, Tharkan JM, Vaideeswar P, Rathinavel A, Narasimhan C, Ayapati DR, Ayub Q, Mehdi SQ, Oppenheimer S, Richards MB, Price AL, Patterson N, Reich D, Singh L, Tyler-Smith C, Thangaraj K (2009) A common MYBPC3 (cardiac myosin binding protein C) variant associated with cardiomyopathies in South Asia. Nat Genet 41(2):187–191. https://doi.org/10.1038/ng.309. Epub 2009 Jan 18

Domanski MJ, Krause-Steinrauf H, Massie BM, Deedwania P, Follmann D, Kovar D, Murray D, Oren R, Rosenberg Y, Young J, Zile M, Eichhorn E, BEST Investigators (2003) A comparative analysis of the results from 4 trials of beta-blocker therapy for heart failure: BEST, CIBIS-II, MERIT-HF, and COPERNICUS. J Card Fail 9(5):354–363. https://doi.org/10.1054/s1071-9164(03)00133-7

Driggin E, Maurer MS (2020) The quintessential form of diastolic heart failure in older adults: wild type transthyretin cardiac amyloidosis. Clin Cardiol 43(2):171–178. https://doi.org/10.1002/clc.23301. Epub 2019 Dec 11

Goldstein DJ, Naka Y, Horstmanshof D, Ravichandran AK, Schroder J, Ransom J, Itoh A, Uriel N, Cleveland JC Jr, Raval NY, Cogswell R, Suarez EE, Lowes BD, Kim G, Bonde P, Sheikh FH, Sood P, Farrar DJ, Mehra MR (2020) Association of clinical outcomes with left ventricular assist device use by bridge to transplant or destination therapy intent: the multicenter study of MagLev technology in patients undergoing mechanical circulatory support therapy with HeartMate 3 (MOMENTUM 3) randomized clinical trial. JAMA Cardiol 5(4):411–419. https://doi.org/10.1001/jamacardio.2019.5323

Harikrishnan S, Sanjay G, Anees T, Viswanathan S, Vijayaraghavan G, Bahuleyan CG, Sreedharan M, Biju R, Nair T, Suresh K, Rao AC, Dalus D, Huffman MD, Jeemon P, Trivandrum Heart Failure Registry (2015) Clinical presentation, management, in-hospital and 90-day outcomes of heart failure patients in Trivandrum, Kerala, India: the Trivandrum Heart Failure Registry. Eur J Heart Fail 17(8):794–800. https://doi.org/10.1002/ejhf.283. Epub 2015 May 23

Harikrishnan S, Bahl A, Roy A, Mishra A, Prajapati J, Nanjappa MC, Sethi R, Guha S, Satheesh S, Chacko M, Ganapathi S, Jeemon P (2019) National Heart Failure Registry, India: design and methods. Indian Heart J 71(16):488–491. https://doi.org/10.1016/j.ihj.2019.12.005. Epub 2020 Jan 3

Huffman MD, Prabhakaran D (2010) Heart failure: epidemiology and prevention in India. Natl Med J India 23(5):283–288

Kato ET, Silverman MG, Mosenzon O, Zelniker TA, Cahn A, Furtado RHM, Kuder J, Murphy SA, Bhatt DL, Leiter LA, McGuire DK, Wilding JPH, Bonaca MP, Ruff CT, Desai AS, Goto S, Johansson PA, Gause-Nilsson I, Johanson P, Langkilde AM, Raz I, Sabatine MS, Wiviott SD (2019) Effect of dapagliflozin on heart failure and mortality in type 2 diabetes mellitus. Circulation 139(22):2528–2536. https://doi.org/10.1161/CIRCULATIONAHA.119.040130. Epub 2019 Mar 18

Kumar R, Singh MC, Singh MC, Ahlawat SK, Thakur JS, Srivastava A, Sharma MK, Malhotra P, Bali HK, Kumari S (2006) Urbanization and coronary heart disease: a study of urban-rural differences in northern India. Indian Heart J 58(2):126–130

Maestre A, Gil V, Gallego J, Aznar J, Mora A, Martín-Hidalgo A (2009) Diagnostic accuracy of clinical criteria for identifying systolic and diastolic heart failure: cross-sectional study. J Eval Clin Pract 15(1):55–61. https://doi.org/10.1111/j.1365-2753.2008.00954.x

Massie BM, Carson PE, McMurray JJ, Komajda M, McKelvie R, Zile MR, Anderson S, Donovan M, Iverson E, Staiger C, Ptaszynska A, I-PRESERVE Investigators (2008) Irbesartan in patients with heart failure and preserved ejection fraction. N Engl J Med 359(23):2456–2467. https://doi.org/10.1056/NEJMoa0805450. Epub 2008 Nov 11

McMurray JJ, Stewart S (2000) Epidemiology, aetiology, and prognosis of heart failure. Heart 83(5):596–602. https://doi.org/10.1136/heart.83.5.596

McMurray JJV, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, Ponikowski P, Sabatine MS, Anand IS, Bělohlávek J, Böhm M, Chiang CE, Chopra VK, de Boer RA, Desai AS, Diez M, Drozdz J, Dukát A, Ge J, Howlett JG, Katova T, Kitakaze M, Ljungman CEA, Merkely B, Nicolau JC, O'Meara E, Petrie MC, Vinh PN, Schou M, Tereshchenko S, Verma S, Held C, DeMets DL, Docherty KF, Jhund PS, Bengtsson O, Sjöstrand M, Langkilde AM, DAPA-HF Trial Committees and Investigators (2019) Dapagliflozin in patients with heart failure and reduced ejection fraction. N Engl J Med 381(21):1995–2008. https://doi.org/10.1056/NEJMoa1911303. Epub 2019 Sept 19

Nair V, Madan H, Sofat S, Ganguli P, Jacob MJ, Datta R, Bharadwaj P, Sarkar RS, Pandit AJ, Nityanand S, Goel PK, Garg N, Gambhir S, George PV, Chandy S, Mathews V, George OK, Talwar KK, Bahl A, Marwah N, Bhatacharya A, Bhargava B, Airan B, Mohanty S, Patel CD, Sharma A, Bhatnagar S, Mondal A, Jose J, Srivastava A, MI3 Trial (2015) Efficacy of stem cell in improvement of left ventricular function in acute myocardial infarction—MI3 Trial. Indian J Med Res 142(2):165–174. https://doi.org/10.4103/0971-5916.164245

O'Meara E, McDonald M, Chan M, Ducharme A, Ezekowitz JA, Giannetti N, Grzeslo A, Heckman GA, Howlett JG, Koshman SL, Lepage S, Mielniczuk LM, Moe GW, Swiggum E, Toma M, Virani SA, Zieroth S, De S, Matteau S, Parent MC, Asgar AW, Cohen G, Fine N, Davis M, Verma S, Cherney D, Abrams H, Al-Hesayen A, Cohen-Solal A, D'Astous M, Delgado DH, Desplantie O, Estrella-Holder E, Green L, Haddad H, Harkness K, Hernandez AF, Kouz S, LeBlanc MH, Lee D, Masoudi FA, McKelvie RS, Rajda M, Ross HJ, Sussex B (2020) CCS/CHFS heart failure guidelines: clinical trial update on functional mitral regurgitation, SGLT2 inhibitors, ARNI in HFpEF, and tafamidis in amyloidosis. Can J Cardiol 36(2):159–169. https://doi.org/10.1016/j.cjca.2019.11.036

Omran AR (1971) The epidemiologic transition: a theory of the epidemiology of population change. Milbank Mem Fund Q 49(4):509–538

Paldino A, De Angelis G, Merlo M, Gigli M, Dal Ferro M, Severini GM, Mestroni L, Sinagra G (2018) Genetics of dilated cardiomyopathy: clinical implications. Curr Cardiol Rep 20(10):83. https://doi.org/10.1007/s11886-018-1030-7

Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, Falk V, González-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GMC, Ruilope LM, Ruschitzka F, Rutten FH, van der Meer P, ESC Scientific Document Group (2016) 2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure: the task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J 37(27):2129–2200. https://doi.org/10.1093/eurheartj/ehw128. Epub 2016 May 20

Schramm R, Zittermann A, Morshuis M, Schoenbrodt M, von Roessing E, von Dossow V, Koster A, Fox H, Hakim-Meibodi K, Gummert JF (2020) Comparing short-term outcome after implantation of the HeartWare® HVAD® and the Abbott® HeartMate 3®. ESC Heart Fail 7(3):908–914. https://doi.org/10.1002/ehf2.12649. Epub 2020 Mar 19

Sethi KK, Dhall A (2011) Optimal utilization of defibrillators and CRT in India—awareness, guidelines and financing—the cardiologist's viewpoint. Indian Heart J 63(4):347–350

Solomon SD, McMurray JJV, Anand IS, Ge J, Lam CSP, Maggioni AP, Martinez F, Packer M, Pfeffer MA, Pieske B, Redfield MM, Rouleau JL, van Veldhuisen DJ, Zannad F, Zile MR, Desai AS, Claggett B, Jhund PS, Boytsov SA, Comin-Colet J, Cleland J, Düngen HD, Goncalvesova E, Katova T, Kerr Saraiva JF, Lelonek M, Merkely B, Senni M, Shah SJ, Zhou J, Rizkala AR, Gong J, Shi VC, Lefkowitz MP, PARAGON-HF Investigators and

Committees (2019) Angiotensin-neprilysin inhibition in heart failure with preserved ejection fraction. N Engl J Med 381(17):1609–1620. https://doi.org/10.1056/NEJMoa1908655. Epub 2019 Sept 1

Vakil RJ (1949) A statistical study of 1281 cases of congestive cardiac failure or myocardial insufficiency in India. Indian Physician 8(10):281–289

Viswanathan SK, Puckelwartz MJ, Mehta A, Ramachandra CJA, Jagadeesan A, Fritsche-Danielson R, Bhat RV, Wong P, Kandoi S, Schwanekamp JA, Kuffel G, Pesce LL, Zilliox MJ, Durai UNB, Verma RS, Molokie RE, Suresh DP, Khoury PR, Thomas A, Sanagala T, Tang HC, Becker RC, Knöll R, Shim W, McNally EM, Sadayappan S (2018) Association of cardiomyopathy with MYBPC3 D389V and MYBPC3Δ25bpIntronic deletion in south Asian descendants. JAMA Cardiol 3(6):481–488. https://doi.org/10.1001/jamacardio.2018.0618

Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL (2013) 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation 128(16):1810–1852. https://doi.org/10.1161/CIR.0b013e31829e8807. Epub 2013 Jun 5

Yusuf S, Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, Michelson EL, Olofsson B, Ostergren J, CHARM Investigators and Committees (2003) Effects of candesartan in patients with chronic heart failure and preserved left-ventricular ejection fraction: the CHARM-Preserved Trial. Lancet 362(9386):777–781. https://doi.org/10.1016/S0140-6736(03)14285-7

Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L, INTERHEART Study Investigators (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 364(9438):937–952. https://doi.org/10.1016/S0140-6736(04)17018-9

Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, Vincent J, Pocock SJ, Pitt B, EMPHASIS-HF Study Group (2011) Eplerenone in patients with systolic heart failure and mild symptoms. N Engl J Med 364(1):11–21. https://doi.org/10.1056/NEJMoa1009492. Epub 2010 Nov 14

IL-10: A Key Molecule in the Mitigation of Heart Failure

13

Ashim K. Bagchi, Akshi Malik, Gauri Akolkar, Adriane Belló-Klein, Neelam Khaper, and Pawan K. Singal

Abstract

Inflammatory mediators play an important role in the pathogenesis of several diseases including heart failure. An appropriate balance between pro-inflammatory and anti-inflammatory cytokines is critical for an optimal functioning of the heart. During ischemia-reperfusion injury, there is an increase in endogenous tumor necrosis factor-α (TNF-α) which plays an important role in initiating and sustaining the inflammatory response as well as cardiac injury. Interleukin 10 (IL-10) is an anti-inflammatory cytokine which tends to suppress many upstream and downstream signaling pathways involved in the pathogenesis of heart failure governed by TNF-α. Here we discuss IL-10, as a mitigating factor and a potential key molecule in heart failure therapy.

Keywords

Anti-inflammatory cytokine · Interleukin-10 · Tumor necrosis factor-α · Heart failure

A. K. Bagchi · A. Malik · P. K. Singal (✉)
Department of Physiology and Pathophysiology, Rady Faculty of Health Sciences, Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, MB, Canada
e-mail: psingal@sbrc.ca

G. Akolkar
Cardiac Function Laboratory, University of Ottawa Heart Institute, Ottawa, ON, Canada

A. Belló-Klein
Laboratório de Fisiologia Cardiovascular, Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

N. Khaper
Northern Ontario School of Medicine, Lakehead University, Thunder Bay, ON, Canada

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_13

13.1 Introduction

Cellular growth, differentiation and function primarily depend upon small protein molecules, also known as cytokines which are secreted by almost every cell in an autocrine, paracrine, juxtacrine and endocrine fashion. These are small molecular weight proteins some of which are highly bioactive and play a critical role in cellular homeostasis. Interleukin 10 (IL-10) is classified as an anti-inflammatory cytokine whose production is mainly controlled by T-cell subsets depending upon the type of stimulation and the signal received by macrophages. Thus, macrophages are considered as the major source of IL-10 production induced by several exogenous and endogenous stimuli such as tumor necrosis factor-α (TNF-α), endotoxins, catecholamines, etc. (Platzer et al. 1995). Interestingly, a systemic release of TNF-α also induces IL-10 via a negative feedback using nuclear factor kappa B cells (NF-κB)-dependent pathway (Barsig et al. 1995; Meisel et al. 1996). IL-10 production in response to TNF-α is an adaptive response to counter the overproduction of TNF-α as well as other inflammatory cytokines such as interferon gamma (IFN-γ) and interleukin-2 (IL-2) (Hess et al. 1997; Lang et al. 2002). IL-10 knockout and antibody treatment studies suggested that lack of IL-10 may exaggerate the inflammatory responses to endotoxemia and peritonitis (van der Poll et al. 1994; Standiford et al. 1995).

IL-10 was originally described as a cytokine synthesis inhibitory factor (CSIF) (Fiorentino et al. 1989; Moore et al. 1990). As shown in Fig. 13.1, IL-10 is a multifunctional cytokine, which acts as an inhibitor, anti-inflammatory, antioxidant molecule, and promotes several cell survival pathways via different mechanisms (Levens et al. 2000; Kaur et al. 2006b; Bagchi et al. 2013, 2017). Homodimeric human IL-10 cytokine is initially transcribed as 178 amino acid (AA) and matured part cleaves as 160 AA with a molecular mass of 37 kDa (Moore et al. 1990).

Cytokines exert their effect in femtomole amounts and trigger inflammatory processes in many disease conditions including cardiovascular diseases. Inflammatory processes involve a number of cells such as macrophages and T-cells for initiating signals in the pathogenesis and/or management of heart failure. IL-10 is not only an immunosuppressant but it also augments receptors to mitigate the effects of TNF-α, IL-1β, and IL-6. Overexpression of these inflammatory cytokinesis known to induce adhesion molecules, chemokines, oxidative stress (OS) molecules, growth factors, prostaglandins, and nitric oxide (NO) (Khaper et al. 2010). We and others have reported that IL-10 antagonizes the TNF–αinduced oxidative stress and cardiomyocyte apoptosis during ischemia-reperfusion injury (Asadullah et al. 2003; Kaur et al. 2006a, b; Dhingra et al. 2009).

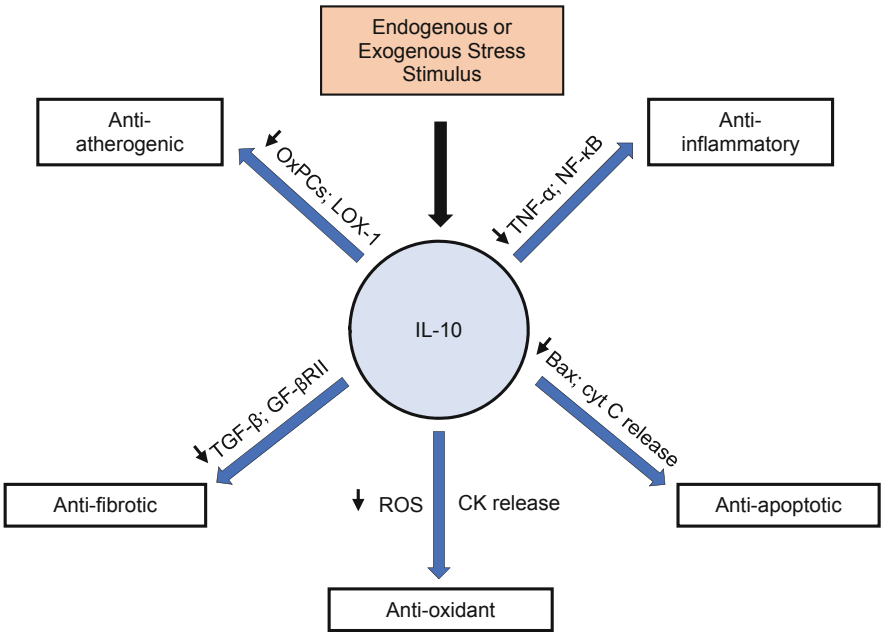


Fig. 13.1 In response to endogenous or exogenous stresses on the heart, interleukin 10 (IL-10) is known to exert a multidimensional protective effect. It can act as an: *Anti-inflammatory* via the negative feedback regulation of nuclear factor-kappa B (NF-κB), thus inhibiting tumor necrosis factor-α (TNF-α); *Antiapoptotic* via inhibiting Bax dimerization and cytochrome (cyt) C release; *Antioxidant* via reducing reactive oxygen species (ROS) and creatine kinase (CK) release; *Antifibrotic* via controlling transforming growth factor-β (TGF-β) and its receptors; and *Anti-atherogenic* via inhibiting endothelial oxidative phospholipids (OxPLs), oxidative phosphatidylcholines (OxPCs) derivative of OxPLs and metabolic responses in a negative regulation of oxidized low-density lipoprotein receptor (LOX-1) by proprotein convertase subtilisin/kexin type 9 (PCSK9). Thus, IL-10 appears to mitigate adversarial effects of different challenges to the heart and offers protection

13.2 Biology of IL-10 in the Heart

Increased endogenous IL-10 has been shown to improve the biological activities as well as contractile function in the heart after growth hormone, dexamethasone, and other steroid treatments (Adamopoulos et al. 2003; El Azab et al. 2002; Giomarelli et al. 2003). IL-10 and TNF-α are the key regulators of T-helper cell type 1 (Th1)/Th2 recruitment in many diseases (Fearon and Locksley 1996; Mosmann and Sad 1996). TNF-α is considered as one of the critical mediators of inflammation leading to heart failure. Results showed that TNF-α also directly induces both the expression and release of IL-10 via a negative feedback loop to suppress TNF-α processing and its synthesis. It may be considered as a key inducer of IL-10 synthesis which, in turn,

effectively suppresses TNF-α in response to endotoxin (van der Poll et al. 1994, 1997). We have shown that IL-10 levels are reduced in severe heart failure subsequent to myocardial infarction (MI) in rats. Both high and low serum levels of IL-10 suggest genetic variations in IL-10 gene expression in different cardiac conditions (Turner et al. 1997; Koch et al. 2001, 2003; Lio et al. 2003). IL-10 gene regulation may be a crucial factor with respect to the final outcome of an inflammatory response (Lio et al. 2003). Increased IL-10 has been associated with a delay in disease progression suggesting its protective role in atherosclerosis (Oslund et al. 1999) and angina (Bolger et al. 2002). Thus, based on in vivo as well as in vitro studies from our and other laboratories, IL-10 can be suggested to mitigate several damaging pathways (Fig. 13.1).

13.3 IL-10 Signaling and Its Regulation in the Heart

Once IL-10 is synthesized and secreted by many cells including cardiomyocytes, it binds to its receptors, present on different cells. Activation of tetrameric transmembrane cytokine receptor, composed of two molecules of high-affinity IL-10R1 and two accessory molecules of low-affinity IL-10R2, initiates pro-survival AKT pathway (Walter 2002). Whereas failure of the receptor activation may lead to loss of function and subsequent antiapoptotic signal activation (Glocker et al. 2011). It also activates downstream pathways which involve Janus kinase 1 (JAK1), Tyrosine Kinase 2 (Tyk2), and signal transducer and activator of transcription 3 (STAT3) dimerization and their nuclear translocation to induce target gene expression (Finbloom and Winestock 1995; Donnelly et al. 1999). STAT3 also promotes transcription of suppressor of cytokine signaling 3 (SOCS3). SOCS3 acts as a negative feedback regulator of IL-10/JAK1/STAT3 signaling and inhibits endotoxin-inducible expression of many pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1 β (Berlato et al. 2002).

13.4 IL-10 in the Mitigation of TNF-α-Induced OS and Apoptosis

It has been suggested that TNF-α may be produced by an increase in wall stress in different loading conditions (Meldrum 1998; Palmieri et al. 2002; Baumgarten et al. 2002). Pro-inflammatory cytokines, including TNF-α, have been shown to contribute to cardiac dysfunction under various pathophysiological conditions associated with heart failure, including ischemia-reperfusion, MI, atherosclerosis, hypertrophy, and acute viral myocarditis (Aukrust et al. 2005; Damås et al. 2001; Mallat et al. 2001; Nishimura et al. 2007; Paulus 2000; Satoh et al. 1999; Sun et al. 2007; Torre-Amione et al. 1996a, b; Yndestad et al. 2007; Blauwet and Cooper 2010). Inflammatory cytokines may modulate cardiovascular function by various mechanisms including altered adrenergic signaling, increases in NO, and alteration of calcium homeostasis and redox imbalance (Ferdinandy et al. 2000; Guggilam et al. 2007; Tatsumi et al. 2000). In the isolated perfused hearts, TNF-α has been shown to cause

a decrease in cardiac function both at the sarcomere as well as cellular levels (Bellahcene et al. 2006) and may contribute directly in the pathogenesis of heart failure (Mann 1996; Torre-Amione et al. 2000; Kaur et al. 2006b).

We and others have shown that heart failure subsequent to MI is associated with an increase in TNF-α (Kaur et al. 2006a: Irwin et al. 1999) and a decrease in IL-10 in the infarcted heart (Kaur et al. 2006a) suggesting that overexpression of TNF-α might provoke cardiovascular complications with increased apoptosis and membrane leakage (Packer 1995) leading to heart failure (Cowie et al. 2000; Levine et al. 1990). Patients with heart failure class III and IV showed a decrease in the plasma level of IL-10 (Stumpf et al. 2008). Thus, an autocrine action of TNF-α, during cardiac dysfunction tends to promote its over-expression and aggravates the disease profile. TNF-α induced increase in reactive oxygen species (ROS), p38 mitogen-activated protein kinase (MAPK), and pro-apoptotic protein Bax results in the development of cardiac cell dysfunction and cell death (Dhingra et al. 2007).

There is now significant evidence that IL-10 has inhibitory action on TNF-α-induced OS and as a result, it suppresses inflammatory pathways as well as apoptosis due to overexpression of TNF-α and contributes to cardiac cells protection (Fig. 13.1) (Kelly et al. 2002; Kaur et al. 2006b). There appears to be a prominent relationship or interaction between TNF-α and IL-10 in heart failure and an imbalance in the levels of the two, in favor of IL-10 may mean heart health and, in the reverse, may result in heart dysfunction. In fact, in isolated adult rat cardiac myocytes, we reported that an imbalance of these two contrasting cytokines in favor of TNF-α leads to increased OS and cardiac myocyte dysfunction which are mitigated by IL-10 (Kaur et al. 2006a; Dhingra et al. 2007, 2009).

Endogenous production of IL-10 showed a critical role in myocardial ischemia/reperfusion injury (Yang et al. 2000). In such conditions, IL-10 not only antagonizes TNF-α-induced changes (Kaur et al. 2006b; Dhingra et al. 2007) but also cuts off the apoptotic signal generated by inhibitor of kappaB (IκB) (Dhingra et al. 2009). Furthermore, we have reported that protective effects of IL-10 are regulated through the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and MAPK which is inhibitory to the TNF-α induced phosphorylation of p38 MAPK activation (Dhingra et al. 2007). Thus, when IL-10 binds to its receptor, not only does it activate the pro-survival signal via the activation of JAK/STAT3 pathway (Dhingra et al. 2007, 2009) but it also inhibits NF-κB–mediated hypertrophic and inflammatory gene expression (Verma et al. 2012).

13.5 IL-10 in the Mitigation of OS-Induced Oxidized Phospholipids During Heart Failure

Oxidative stress appears to be a major biological consequence of all cardiac disease pathologies, where excess amount of ROS is produced against the antioxidant defense. The major source of ROS production during cardiac cell injury is through disruption of mitochondrial function and an increase in different enzyme activities including NADPH oxidase, xanthine oxidase, and uncoupling of nitric oxide

synthase (NOS) (Takimoto and Kass 2007). Studies have shown that the OS-induced inflammatory response resulting from MI serves to further exacerbate myocardial injury, leading to deleterious remodeling of the heart (Kaur et al. 2006b; Khaper et al. 2010). Mitochondrial ROS are known to activate matrix metalloproteinases and excess collagen formation, accompanied by increase apoptosis and hypertrophy (Pacher et al. 2005; Sabri et al. 2003). Importantly, self-amplification of the inflammatory signal transduction pathways may also lead to depressed cardiac contractility (Adamy et al. 2007; Bradham et al. 2002; Haudek et al. 2007; Li et al. 2000; Morimoto et al. 2006; Nian et al. 2004).

Moreover, ROS causes oxidation of membrane phospholipids (OxPLs) and the formation of oxidative phosphatidylcholines (OxPCs) (Allen et al. 2013; Ganguly et al. 2018; Samhan-Arias et al. 2012). These OxPCs, particularly fragmented, are implicated in various inflammatory diseases including atherosclerosis and ischemia/reperfusion (I/R) injury in the heart (Yeang et al. 2019; Bagchi et al. 2020). We have shown that IL-10 inhibited OS-induced OxPCs compounds by 50%. Furthermore, heatmap data generated from 80 different species of OxPCs suggested that 24 (30%) of OxPCs were modulated significantly (log2 Fold Change; $P < 0.001$) by IL-10, and 6 of these OxPCs were fragmented. Moreover, we also reported a cardiomyocyte-specific modulation in these fragmented OxPCs by IL-10, in that PAzPC (1-palmityl-2-azelyl-*sn*-glycero-3-phosphocholine), a homologue of 1-palmityl-2-(5-glutaryl)-*sn*-glycero-3-phosphocholine (PGPC) was maximally modulated by IL-10 (FDR < 0.0006) (Bagchi et al. 2020). It appears that PAzPC induces a release of the mitochondrial cytochrome c (Cyt c) and promotes apoptosis-inducing factor (AIF) (Kagan et al. 2009; Bagchi et al. 2020). Mitochondrial dysfunction and apoptosis due to OxPCs are favorably influenced by hexadecylazeloylglycerophosphocoline (HAzPC), a homologue of PAzPC (Chen et al. 2007). The latter was affected by IL-10 treatment, suggesting that IL-10 may decrease PAzPC and control mitochondrial release of Cyt c and troponin 1c (Bagchi et al. 2020).

Another fragmented OxPC, SAzPC, a homologue of 1-stearoyl-2-glutaroyl-snglycero-3-phosphocholine (SGPC) as a second major fragmented OxPCs compound in the heart also decreased by IL-10. Since SAzPC levels were increased only in I/R hearts and not in isolated cardiomyocytes, it is suggested to be a non-cardiomyocyte-specific heart-response during I/R injury which may have promoted TGF-β-mediated fibrosis (Bagchi et al. 2020). SAzPC induces platelet activation causing thrombosis (Göpferta et al. 2005; Barreto et al. 2021). Under these stress conditions such as I/R and exposure to 1-palmityl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine (POVPC), we have also reported an upregulation of LOX-1, a receptor for oxidized LDL (low-density lipoprotein) (Bagchi et al. 2020). It is known that prolonged oxidation of POVPC gives rise to PGPC and further oxidation synthesizes PAzPC as an irreversible end-product (Salomon 2012) which promoted toll-like receptor-2 (TLR-2). We proposed that PAzPC as a "danger species" recognizes TLR2 and triggers downstream OS-induced inflammation leading to cardiomyocyte death. Antioxidant property of IL-10 may have reduced prolonged oxidation of OxPLs and inhibited PAzPC synthesis in cardiomyocytes as well as

SAzPC in other cells of the heart. These two major species in I/R injury condition may have worsened the OS response-mediated inflammation and cardiac cell death leading to heart damage which was mitigated by IL-10.

13.6 IL-10 in the Mitigation of Lipid Metabolic Responses

In response to OS, the body triggers tissue-specific metabolic responses to maintain normal physiology for survival. A robust fatty acid oxidation is required to fulfill the energy needed by an adult heart muscle (Lopaschuk 2017). The rate of fatty acid oxidation depends on many physiological functions including the rate of acyl translocation across the mitochondrial membrane and the rate of acetyl-CoA oxidation by the citric acid cycle (Goldberg et al. 2012). Under stressful conditions, oxidation of fatty acid is impaired and thus heart muscle accumulates OxPLs leading to cardiac lipotoxicity or metabolic dilated cardiomyopathy (Goldberg et al. 2012). Dysregulation of lipid metabolism during cardiac ischemia is due to either increased lipid uptake via very-low-density lipoprotein (VLDL) receptor activation (Perman et al. 2011) or decreased glucose oxidation.

Increased lipid uptake induces mitochondrial dysfunction via the production of ROS and RNS, which causes myocyte apoptosis and hypertrophy (Takimoto and Kass 2007). On the other hand, ROS causes oxidation of membrane phospholipids, resulting in the accumulation of OxPLs during fatty acid metabolism and leads to the formation of OxPCs (Goldberg et al. 2012). These OxPCs bind to scavenger receptors such as CD36 and lectin-like LOX1 and promote downstream metabolic pathways to regulate cardiac remodeling (Chen et al. 2003; Barreto et al. 2021). In patients with a deficiency of long-chain fatty acid transport during CD36 deficiency, there is increase in myocardial glucose use (Fukuchi et al. 1999). In vitro exposure of isolated cardiomyocytes to 5 μM POVPC promoted LOX-1 expression (Bagchi et al. 2020). It is suggested that LOX-1 interferes with lipid metabolism by promoting oxLDL uptake (Kume et al. 2000). In general, LOX-1 promotes proprotein convertase subtilisin/kexin type 9 (PCKS9) whereas in cardiomyocyte, we noticed that increased LOX-1 is independent of PCSK9 activation. IL-10 was able to overcome PCSK9 inhibition and promoted PCSK9 and LOX-1, suggesting that IL-10 negatively regulates LOX-1 expression via PCSK9. Also, IL-10 mediated PCSK9 signals are required for LOX-1 inhibition, confirmed by using PCSK9 inhibitor (Bagchi et al. 2020). During phospholipid oxidation, there was an increased expression of sterol regulatory element-binding protein 1c (SREBP1c) which was inhibited by IL-10 suggesting that PCSK9 might differently regulate SREBP1c and control downstream LOX-1. It is known that SREBP1c plays a vital role in lipid synthesis as well as its uptake (Glerup et al. 2017; Chen et al. 2007; Xiao and Song 2013) and thus, its inhibition by IL-10 may be important in the treatment of lipid metabolic disorders. LOX-1 upregulation has been reported to cause endothelial dysfunction, increase platelet adhesion and cardiac fibrosis as well as apoptosis (Barreto et al. 2021).

13.7 IL-10 in Innate Signaling

Inflammatory process in response to myocardial injury is mainly controlled by cytokines. Most of the cytokines follow innate signaling pathway via patterns recognition receptors called Toll-like receptors (TLRs) and its adaptor molecule myeloid differentiation gene factor 88 (MyD88) (Bagchi et al. 2013). These receptors recognize conserved molecular motifs, pathogen-associated molecular patterns, or endogenous molecules, secreted by damaged cells known as damage-associated molecular patterns (DAMP). Upregulation of TLRs, specifically TLR2 and TLR4 which influence cardiac function is regulated through the downstream activation of NF-κB (Yu and Feng 2018; Gullestad et al. 2012; Frantz et al. 2001). Nevertheless, details of such an innate response are still unclear. We have shown that IL-10 activates TLR4 and innate responses in order to improve cardiomyocyte survival (Bagchi et al. 2013). Thus, IL-10 might be considered as an important immunoregulatory molecules of the innate system to promote cardiac health (Fig. 13.1).

Activation of the TLR2 eventually leads to a reduction in cardiac function through Interleukin-1 receptor-associated kinase-M (IRAK-M) dependent NF-κB activation (Bagchi et al. 2017; Zlobine et al. 2016). TLR2 activation, in response to OS-induced TNF-α-mediated changes in TNF-α receptor-associated death domain (TRADD) as well as Fas-associated death domain (FADD) in cardiomyocytes, is detrimental for the heart (Bagchi et al. 2017). However, IL-10 modulated TLR2-mediated downstream changes in the heart during I/R injury (Bagchi et al. 2017). Interestingly, activation of TLR4 by IL-10 tended to promote a survival pathway via deactivation of OS-induced oxidized phospholipids (OxPLs) metabolite production as well as TLR2 innate signaling pathways (Bagchi et al. 2017).

13.8 Cytokine Imbalance and Cardiovascular Complications

Inflammation is an intricate signaling process where a series of events are triggered by pro-inflammatory cytokines or by metabolic mediators. Many pro-inflammatory cytokines share overlapping biological actions. TNF-α and IL-1β are two main pro-inflammatory cytokines that are involved in almost every pathological condition and trigger downstream pathways to alter cellular physiological balance. Increased TNF-α in mice contributes in dilated cardiomyopathy as well as it is associated with increased fibrosis in the heart (Sun et al. 2007). MI in mice showed an increase in both TNF-αand IL-10 levels (Yao et al. 2008). In contrast, MI in the IL-10 −/− mice showed an increase in infarct size as well as cellular apoptosis (Yang et al. 2000). Exogenous administration of IL-10 in these MI conditions improved cardiac function and reduced inflammation (Jung et al. 2017). Patients with chronic congestive heart failure showed a low IL-10 to TNF-α ratio (Stumpf et al. 2008). Imbalance between IL-10 and TNF-α has been shown to correlate with stable and unstable angina (Waehre et al. 2002). IL-1β has also been shown to promote myocyte hypertrophy with preserved LV systolic function (Zhang et al. 2011). IL-10

suppresses the inflammatory response and contributes to improved LV function and remodeling in acute MI models (Frangogiannis et al. 2000). Improved heart function is associated with an increase in IL-10/TNF-α ratio (Stumpf et al. 2008; Waehre et al. 2002). IL-10 restricted the deleterious effects of TNF-α by reducing ROS generation considering that this molecule also functions as an antioxidant (Kaur et al. 2006a; Dhingra et al. 2007).

Modulation of OxPCs, specifically PAzPC and SAzPC, suggested that these molecules have the potential to mitigate OS during I/R injury via negative feedback regulation of PCSK9 and inhibition of LOX-1 receptors (Bagchi et al. 2020). Inhibition of IL-10 receptors abrogated the beneficial downstream effect of TLR4 leading to apoptosis (Bagchi et al. 2013). Anti-inflammatory property of IL-10 is regulated through a feedback innate signaling mechanism. In continuation, IL-10 has been shown to reduce TNF-α associated changes in TRADD and FADD and reduction in Bax/Bcl-xl ratio and enzyme poly ADP ribose polymerase (PARP) cleavage without change in caspase 3. Thus, IL-10 does function as an antiapoptotic molecule (Bagchi et al. 2017). An in vitro study also suggested that IL-10 treatment maintained cytokine homeostasis by elevating IL-1β even after removal of IL-10 stimulation (Bagchi et al. 2013). It is assumed that IL-1β may trigger another pro-inflammatory pathway to activate endogenous IL-10 by a selective cell recruitment, but it needs to be studied.

In conclusion, it is suggested that IL-10 is one of the key mediators of cardiac repair and survival in stressful conditions that are promoted by both inflammatory and oxidative processes leading to cell death pathways (Fig. 13.1). Any compromise in IL-10 response might result in an OS-inflammatory environment, which has been suggested to play a significant role in the pathogenesis of heart failure.

Acknowledgments This study was supported by operating grants from the Heart and Stroke Foundation of Canada and Molson Women's Heart Health Program. P.K. Singal is the holder of the Naranjan Dhalla Chair in Cardiovascular Research supported by the St. Boniface Hospital Foundation.

References

Adamopoulos S, Parissis JT, Paraskevaidis I, Karatzas D, Livanis E, Georgiadis M, Karavolias G, Mitropoulos D, Degiannis D, Kremastinos DT (2003) Effects of growth hormone on circulating cytokine network, and left ventricular contractile performance and geometry in patients with idiopathic dilated cardiomyopathy. Eur Heart J 24:2186–2196. https://doi.org/10.1016/S0195-668X(03)00480-9

Adamy C, Mulder P, Khouzami L, Andrieu-Abadie N, Defer N, Candiani G, Pavoine C, Caramelle P, Souktani R, Le Corvoisier P, Perier M, Kirsch M, Damy T, Berdeaux A, Levade T, Thuillez C, Hittinger L, Packer F (2007) Neutral Sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats. J Mol Cell Cardiol 43:344–353. https://doi.org/10.1016/j.yjmcc.2007.06.010

Allen D, Hasanally D, Ravandi A (2013) Role of oxidized phospholipids in cardiovascular pathology. Clin Lipidol 8:205–215. https://doi.org/10.2217/CLP.13.13

Asadullah K, Sterry W, Volk HD (2003) Interleukin-10 therapy—review of a new approach. Pharmacol Rev 55:241–269. https://doi.org/10.1124/pr.55.2.4

Aukrust P, Gullestad L, Ueland T, Damas JK, Yndestad A (2005) Inflammatory and anti-inflammatory cytokines in chronic heart failure: potential therapeutic implications. Ann Med 37:74–85. https://doi.org/10.1080/07853890510007232

Bagchi AK, Sharma AK, Dhingra S, Ludke ARL, Singal PK (2013) Interleukin-10 activates Toll-like receptor4 and MyD88 adaptor molecule for cardiomyocyte survival. Cytokine 61:304–314. https://doi.org/10.1016/j.cyto.2012.10.013

Bagchi AK, Akolkar G, Mandal S, Ayyappan P, Yang X, Singal PK (2017) Toll-like receptor 2 dominance over toll-like receptor 4 in stressful conditions for its detrimental role in the heart. Am J Physiol Heart Circ Physiol 312:H1238–H1247. https://doi.org/10.1152/ajpheart.00800.2016

Bagchi AK, Surendran A, Malik A, Jassal DS, Ravandi A, Singal PK (2020) IL-10 attenuates OxPCs-mediated lipid metabolic responses in ischemia reperfusion injury. Sci Rep 10:12120. https://doi.org/10.1038/s41598-020-68995-z

Barreto J, Karathanasis SK, Remaley A, Sposito AC (2021) Role of LOX-1 (Lectin-Like Oxidized Low-Density Lipoprotein Receptor 1) as a cardiovascular risk predictor: mechanistic insight and potential clinical use. Arterioscler Thromb Vasc Biol 41:153–166. https://doi.org/10.1161/ATVBAHA.120.315421

Barsig J, Küsters S, Vogt K, Vogt H-D, Tiegs G, Wendel A (1995) Lipopolysaccharide-induced interleukin-10 in mice: role of endogenous tumor necrosis a. Eur J Immunol 25:2888–2893. https://doi.org/10.1002/eji.1830251027

Baumgarten G, Knuefermann P, Kalra D, Gao F, Taffet GE, Michael L, Blackshear PJ, Carballo E, Sivasubramanian N, Mann DL (2002) Load-dependent and independent regulation of proinflammatory cytokine and cytokine receptor gene expression in the adult mammalian heart. Circulation 105:2192–2197. https://doi.org/10.1161/01.cir.0000015608.37608.18

Bellahcene M, Jacquet S, Cao XB, Tanno M, Haworth RS, Layland J, Kabir AM, Gaestel M, Davis RJ, Flavell RA, Shah AM, Avkiran M, Marber MS (2006) Activation of p38 mitogen-activated protein kinase contributes to the early cardiodepressant action of tumor necrosis factor. J Am Coll Cardiol 48:545–555. https://doi.org/10.1016/j.jacc.2006.02.072

Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F (2002) Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol 168:6404–6411. https://doi.org/10.4049/jimmunol.168.12.6404

Blauwet LA, Cooper LT (2010) Myocarditis. Prog Cardiovasc Dis 52:274–288. https://doi.org/10.1016/j.pcad.2009.11.006

Bolger AP, Sharma R, von Haehling S, Doehner W, Oliver B, Rauchhaus M, Coats AJS, Adcock IM, Anker SD (2002) Effect of interleukin-10 on the production of tumor necrosis factor-alpha by peripheral blood mononuclear cells from patients with chronic heart failure. Am J Cardiol 90:384–389. https://doi.org/10.1016/s0002-9149(02)02494-3

Bradham WS, Moe G, Wendt KA, Scott AA, Konig A, Romanova M, Naik G, Spinale FG (2002) TNF-alpha and myocardial matrix metalloproteinase in heart failure: relationship to LV remodeling. Am J Physiol Heart Circ Physiol 282:1288–1295. https://doi.org/10.1152/ajpheart.00526.2001

Chen Q, Reis SE, Kammerer C, Craig WY, LaPierre SE, Zimmer EL, McNamara DM, Pauly DF, Sharaf B, Holubkov R, Merz CNB, Sopko G, Bontempo F, Kamboh MI (2003) Genetic variation in lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) gene and the risk of coronary artery disease. Circulation 107:3146–3151. https://doi.org/10.1161/01.CIR.0000074207.85796.36

Chen R, Yang L, McIntyre T (2007) Cytotoxic phospholipid oxidation products. Cell death from mitochondrial damage and the intrinsic caspase cascade. J Biol Chem 282:24842–24850. https://doi.org/10.1074/jbc.M702865200

Cowie MR, Wood DA, Coats AJ, Thompson SG, Suresh V, Poole-Wilson PA, Sutton GC (2000) Survival of patients with a new diagnosis of heart failure: a population based study. Heart 83: 505–510. https://doi.org/10.1136/heart.83.5.505

Damås JK, Gullestad L, Aass H, Simonsen S, Fjeld JG, Wikeby L, Ueland T, Eiken HG, Froland SS, Aukrust P (2001) Enhanced gene expression of chemokines and their corresponding receptors in mononuclear blood cells in chronic heart failure—modulatory effect of intravenous immunoglobulin. J Am Coll Cardiol 38:187–193. https://doi.org/10.1016/s0735-1097(01)01335-3

Dhingra S, Sharma AK, Singla DK, Singal PK (2007) P38 and ERK1/2 MAPKs mediate the interplay of TNF-α and IL-10 in regulating oxidative stress and cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 293:3524–3531. https://doi.org/10.1152/ajpheart.00919.2007

Dhingra S, Sharma AK, Arora RC, Slezak J, Singal PK (2009) IL-10 attenuates TNF-α-induced NFkB pathway activation and cardiomyocyte apoptosis. Cardiovasc Res 82:59–66. https://doi.org/10.1093/cvr/cvp040

Donnelly RP, Dickensheets H, Finbloom DS (1999) The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. J Interferon Cytokine Res 19:563–573. https://doi.org/10.1089/107999099313695

El Azab SR, Rosseel PM, de Lange JJ, Groeneveld ABJ, Strik R, van Wijk EM, Scheffer GJ (2002) Dexamethasone decreases the pro- to anti-inflammatory cytokine ratio during cardiac surgery. Br J Anaesth 88:496–501. https://doi.org/10.1093/bja/88.4.496

Fearon DT, Locksley RM (1996) The instructive role of innate immunity in the acquired immune response. Science 272:50–53. https://doi.org/10.1126/science.272.5258.50

Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R (2000) Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ Res 87:241–247. https://doi.org/10.1161/01.res.87.3.241

Finbloom DS, Winestock KD (1995) IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. J Immunol 155:1079–1090. PMID: 7543512

Fiorentino DF, Bond MW, Mosmann TR (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170:2081–2095. https://doi.org/10.1084/jem.170.6.2081

Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, Entman ML (2000) IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J Immunol 165:2798–2808. https://doi.org/10.4049/jimmunol.165.5.2798

Frantz S, Kelly RA, Bourcier T (2001) Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. J Biol Chem 276(7):5197–5203. https://doi.org/10.1074/jbc.M009160200

Fukuchi K, Nozaki S, Yoshizumi T, Hasegawa S, Uehara T, Nakagawa T, Kobayashi T, Tomiyama Y, Yamashita S, Matsuzawa Y, Nishimura T (1999) Enhanced myocardial glucose use in patients with a deficiency in long-chain fatty acid transport (CD36 deficiency). J Nucl Med 40:239–243. PMID: 10025829

Ganguly R, Hasanally D, Stamenkovic A, Maddaford TG, Chaudhary R, Pierce GN, Ravandi A (2018) Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. Mol Cell Biochem 437:163–175. https://doi.org/10.1007/s11010-017-3104-z

Giomarelli P, Scolletta S, Borrelli E, Biagioli B (2003) Myocardial and lung injury after cardiopulmonary bypass: role of interleukin (IL)-10. Ann Thorac Surg 76:117–123. https://doi.org/10.1016/S0003-4975(03)00194-2

Glerup S, Schulz R, Laufs U, Schlüter KD (2017) Physiological and therapeutic regulation of PCSK9 activity in cardiovascular disease. Basic Res Cardiol 112:32. https://doi.org/10.1007/s00395-017-0619-0

Glocker EO, Kotlarz D, Klein C, Shah N, Grimbacher B (2011) IL-10 and IL-10 receptor defects in humans. Ann N Y Acad Sci 1246:102–107. https://doi.org/10.1111/j.1749-6632.2011.06339.x

Goldberg IJ, Trent CM, Schulze PC (2012) Lipid metabolism and toxicity in the heart. Cell Metab 15:805–812. https://doi.org/10.1016/j.cmet.2012.04.006

Göpferta MS, Siedlerc F, Siessa W, Sellmayera A (2005) Structural identification of oxidized acyl-phosphatidylcholines that induce platelet activation. J Vasc Res 42:120–132. https://doi.org/10.1159/000083461

Guggilam A, Haque M, Kerut EK, McIlwain E, Lucchesi P, Seghal I, Fransis J (2007) TNF-alpha blockade decreases oxidative stress in the paraventricular nucleus and attenuates sympathoexcitation in heart failure rats. Am J Physiol Heart Circ Physiol 293:H599–H609. https://doi.org/10.1152/ajpheart.00286.2007

Gullestad L, Ueland T, Vinge LE, Finsen A, Yndestad A, Aukrust P (2012) Inflammatory cytokines in heart failure: mediators and markers. Cardiology 122:23–35. https://doi.org/10.1159/000338166

Haudek SB, Taffet GE, Schneider MD, Mann DL (2007) TNF provokes cardiomyocytes apotosis and cardiac remodeling through activation of multiple cell death pathways. J Clin Invest 117:2692–2701. https://doi.org/10.1172/JCI29134

Hess PJ, Seeger JM, Huber TS, Welborn MB, Martin TD, Harward TR, Duschek S, Edwards PD, Solorzano CC, Copeland EM, Moldawer LL (1997) Exogenously administrated interleukin-10 decreases pulmonary neutrophil infiltration in a tumor necrosis factor-dependent murine model of acute visceral ischaemia. J Vasc Surg 26:113–118. https://doi.org/10.1016/S0741-5214(97)70154-X

Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, Dawood F, Wen WH, Shou Z, Liu P (1999) Tissue expression and immunolocalization of tumor necrosis factor-alpha in postinfarction dysfunctional myocardium. Circulation 23:1492–1498. https://doi.org/10.1161/01.cir.99.11.1492

Jung M, Ma Y, Iyer RP, DeLeon-Pennell KY, Yabluchanskiy A, Garrettt MR, Lindsey ML (2017) IL-10 improves cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation. Basic Res Cardiol 112:33. https://doi.org/10.1007/s00395-017-0622-5

Kagan VE, Bayir A, Bayir H, Stoyanovsky D, Borisenko GG, Tyurina YY, Wipf P, Atkinson J, Greenberger JS, Chapkin RS, Belikova NA (2009) Mitochondria-targeted disruptors and inhibitors of cytochrome c/cardiolipin peroxidase complexes: a new strategy in anti-apoptotic drug discovery. Mol Nutr Food Res 53:104–114. https://doi.org/10.1002/mnfr.200700402

Kaur K, Sharma AK, Singal PK (2006a) Significance of changes in TNF-alpha and IL-10 levels in progression of heart failure subsequent to myocardial infarction. Am J Physiol Heart Circ Physiol 291:H106–H113. https://doi.org/10.1152/ajpheart.01327.2005

Kaur K, Sharma AK, Dhingra S, Singal PK (2006b) Interplay of TNF-α and IL-10 in regulating oxidative stress in isolated adult cardiac myocytes. J Mol Cell Cardiol 41:1023–1030. https://doi.org/10.1016/j.yjmcc.2006.08.005

Kelly A, Lynch A, Vereker E, Nolan Y, Queenan P, Whittaker E, O'Neill L, Lynch M (2002) The anti-inflammatory cytokine, interleukin (IL)-10, blocks the inhibitory effect of IL-1β on long term potentiation: a role for JNK. J Biol Chem 276:45564–45572. https://doi.org/10.1074/jbc.M108757200

Khaper N, Bryan S, Dhingra S, Singal R, Bajaj A, Pathak CM, Singal PK (2010) Targeting the vicious inflammation-oxidative stress cycle for the management of heart failure. Antioxid Redox Signal 13:1033–1049. https://doi.org/10.1089/ars.2009.2930

Koch W, Kastrati A, Bottiger C, Mehilli J, von Beckerath N, Schomig A (2001) Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis 159:137–144. https://doi.org/10.1016/s0021-9150(01)00467-1

Koch W, Tiroch K, von Beckerath N, Schomig A, Kastrati A (2003) Tumor necrosis factor-alpha, lymphotoxin-alpha, and interleukin-10 gene polymorphisms and restenosis after coronary artery stenting. Cytokine 24:161–171. https://doi.org/10.1016/j.cyto.2003.08.004

Kume N, Moriwaki H, Kataokaetal H (2000) Inducible expression of LOX-1, a novel receptor for oxidized ldl, in macrophages and vascular smooth muscle cells. In: Numanoand F, Gimbrone

MA (eds) Atherosclerosis V: the fifth Saratoga conference, vol 902. New York Academy of Sciences, New York, pp 323–327. https://doi.org/10.1007/978-4-431-68424-4_42

Lang R, Rutschman RL, Greaves DR, Murray PJ (2002) Autocrine deactivation of macrophages in transgenic mice constitutively overexpressing IL-10 under control of the human CD68 promoter. J Immunol 168:3402–3411. https://doi.org/10.4049/jimmunol.168.7.3402

Levens JM, Gordon J, Gregory CD (2000) Micro-environmental factors in the survival of human B-lymphoma cells. Cell Death Differ 7:59–69. https://doi.org/10.1038/sj.cdd.4400636

Levine B, Kalman J, Mayer L, Fillit HM, Packer M (1990) Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 323:236–241. https://doi.org/10.1056/NEJM199007263230405

Li X, Moody MR, Engel D, Walker S, Clubb FJ, Sivasubramanian N, Mann DL, Reid MB (2000) Cardiac-specific overexpression of tumor factor-alpha causes oxidative stress and contractile dysfunction in mouse diaphragm. Circulation 102:1690–1696. https://doi.org/10.1161/01.cir.102.14.1690

Lio D, Scola L, Crivello A, Colonna-Romano G, Candore G, Bonafe M, Cavallone L, Marchegiani F, Olivieri F, Franceschi C, Caruso C (2003) Inflammation, genetics, and longevity: further studies on the protective effects in men of IL-10-1082 promoter SNP and its interaction with TNF-alpha-308 promoter SNP. J Med Genet 40:296–299. https://doi.org/10.1136/jmg.40.4.296

Lopaschuk GD (2017) Metabolic modulators in heart disease: past, present, and future. Can J Cardiol 33:838–849. https://doi.org/10.1016/j.cjca.2016.12.013

Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, Seidler T, Hasenfuss G, Chvatchko Y, Shah AM, Tedgui A (2001) Evidence or altered interleukin 18 (IL)-18 pathway in Blum A, Miller H. Pathophysiological role of cytokine in congestive heart failure. Ann Rev Med 52:15–27. https://doi.org/10.1096/fj.04-2426fje

Mann DL (1996) The effect of tumor necrosis factor-alpha on cardiac structure and function: a tale of two cytokines. J Card Fail 2:S165–S172. https://doi.org/10.1016/s1071-9164(96)80073-x

Meisel C, Vogt K, Platzer C, Randow F, Liebenthal C, Volk HD (1996) Differential regulation of monocytic tumor necrosis factor-alpha and interleukin-10 expression. Eur J Immunol 26:1580–1586. https://doi.org/10.1002/eji.1830260726

Meldrum DR (1998) Tumor necrosis factor in the heart. Am J Physiol 274:R577–R595. https://doi.org/10.1152/ajpregu.1998.274.3.R577

Moore KW, Vieira P, Fiorentino DF, Trounstine ML, Khan TA, Mosmann TR (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science 248:1230–1234. https://doi.org/10.1126/science.2161559

Morimoto H, Takahashi M, Izawa A, Ise H, Hongo M, Kolattukudy PE, Ikeda U (2006) Cardiac overexpression of monocyte chemoattractant protein-1 in transgenic mice prevents cardiac dysfunction and remodeling after myocardial infarction. Circ Res 99:891–899. https://doi.org/10.1161/01.RES.0000246113.82111.2d

Mosmann TR, Sad S (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 17:138–146. https://doi.org/10.1016/0167-5699(96)80606-2

Nian M, Lee P, Khaper N, Liu PP (2004) Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 94:1543–1553. https://doi.org/10.1161/01.RES.0000130526.20854.fa

Nishimura Y, Inoue T, Nitto T, Morooka T, Node K (2007) Increased interleukin-13 levels in patients with chronic heart failure. Int J Cardiol 131:421–423. https://doi.org/10.1016/j.ijcard.2007.07.128

Oslund LJP, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI (1999) Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol 19:2847–2853. https://doi.org/10.1161/01.atv.19.12.2847

Pacher P, Schulz R, Liaudet L, Szabo C (2005) Nitrosative stress and pharmacological modulation of heart failure. Trends Pharmacol Sci 26:302–310. https://doi.org/10.1016/j.tips.2005.04.003

Packer M (1995) Is tumor necrosis factor an important neurohormonal mechanism in chronic heart failure? Circulation 92:1379–1382. https://doi.org/10.1161/01.cir.92.6.1379

Palmieri EA, Benincasa G, Di Rella F, Casaburi C, Monti MG, De Simone G, Chiariotti L, Palombini L, Bruni CB, Sacca L, Cittadini A (2002) Differential expression of TNF-alpha, IL-6, and IGF-1 by graded mechanical stress in normal rat myocardium. Am J Phyisol Heart Circ Physiol 282:H926–H934. https://doi.org/10.1152/ajpheart.00436.2001

Paulus WJ (2000) Cytokines and heart failure. Heart Fail Monit 1:50–60. PMID: 12634874

Perman JC, Boström P, Lindbom M et al (2011) The VLDL receptor promotes lipotoxicity and increases mortality in mice following an acute myocardial infarction. J Clin Invest 121:2625–2640. https://doi.org/10.1172/JCI43068

Platzer C, Meisel C, Vogt K, Platzer M, Volk HD (1995) Up-regulation of monocytic IL-10 by tumor necrosis factor-alpha and cAMP elevating drugs. Int Immunol 7:517–7523. https://doi.org/10.1093/intimm/7.4.517

Sabri A, Hughie HH, Lucchesi PA (2003) Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. Antioxid Redox Signal 5:731–740. https://doi.org/10.1089/152308603770380034

Salomon RG (2012) Structural identification and cardiovascular activities of oxidized phospholipids. Circ Res 111:930–946. https://doi.org/10.1161/CIRCRESAHA.112.275388

Samhan-Arias AK, Ji J, Demidova OM, Sparvero LJ, Feng W, Tyurin V, Tyurina YY, Epperly MW, Shvedova AA, Greenberger JS, Bayir H, Kagan VE, Amoscato AA (2012) Oxidized phospholipids as biomarkers of tissue and cell damage with a focus on cardiolipin. Biochim Biophys Acta 1818:2413–2436. https://doi.org/10.1016/j.bbamem.2012.03.014

Satoh M, Nakamura M, Saitoh H, Maesawa C, Segawa I, Tashiro A, Hiramori K (1999) Tumor necrosis factor-alpha-converting enzyme and tumor necrosis factor-alpha in human dilated cardiomyopathy. Circulation 99:3260–3265. https://doi.org/10.1161/01.cir.99.25.3260

Standiford TJ, Strieter RM, Lukacs NW, Kunkel SL (1995) Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. J Immunol 155:2222–2229

Stumpf C, Seybold K, Petzi S, Wasmeier G, Raaz D, Yilmaz A, Anger T, Daniel WG, Garlichs CD (2008) Interleukin-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction. Eur J Heart Fail 10:733–739. https://doi.org/10.1016/j.ejheart.2008.06.007

Sun M, Chen M, Dawood F, Zurawska U, Li JY, Parker T, Kassisi Z, Kirshenbaum LA, Arnold M, Khokha R, Liu PP (2007) Tumor necrosis factor-alpha mediates cardiac remodeling and ventricular dysfunction after pressure overload state. Circulation 115:1398–1407. https://doi.org/10.1161/CIRCULATIONAHA.106.643585

Takimoto E, Kass DA (2007) Role of oxidative stress in cardiac hypertrophy and remodeling. Hypertension 49:241–248. https://doi.org/10.1161/01.HYP.0000254415.31362.a7

Tatsumi T, Matoba S, Kawahara A, Keira N, Shiraishi J, Akashi K, Kobara M, Tanaka T, Katamura M, Nakagawa C, Ohta B, Shirayama T, Takeda K, Asayama J, Fliss H, Nakagawa M (2000) Cytokine-induced nitric oxide production inhibits mitochondrial energy production and impairs contractile function in rat cardiac myocytes. J Am Coll Cardiol 35:1338–1346. https://doi.org/10.1016/s0735-1097(00)00526-x

Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB, Mann DL (1996a) Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). J Am Coll Cardiol 27:1201–1206. https://doi.org/10.1016/0735-1097(95)00589-7

Torre-Amione G, Kapadia S, Lee J, Durand JB, Bies RD, Young JB, Mann DL (1996b) Tumor necrosis factor-alpha and tumor necrosis factor receptors in the failing human heart. Circulation 93:704–711. https://doi.org/10.1161/01.cir.93.4.704

Torre-Amione G, Vooletich MT, Farmer JA (2000) Role of tumor necrosis factor-alpha in the progression of heart failure: therapeutic implications. Drugs 59:745–751. https://doi.org/10.2165/00003495-200059040-00002

Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV (1997) An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 24:1–8. https://doi.org/10.1111/j.1365-2370.1997.tb00001.x

van der Poll T, Jansen J, Levi M, ten-Cate H, ten-Cate JW, van Deventer SJ (1994) Regulation of interleukin-10 release by tumor necrosis factor-alpha in humans and chimpanzees. J Exp Med 180:1985–1988. https://doi.org/10.1084/jem.180.5.1985

van der Poll T, Jansen PM, Montegut WJ, Braxton CC, Salvano SE, Stackpole SA, Smith SR, Swanson SW, Hack CE, Lowry SF, Moldawer LL (1997) Effects of IL-10 on systemic inflammatory responses during sublethal primate endotoxemia. J Immunol 158:1971–1975. PMID: 9029140

Verma SK, Krishnamurthy P, Barefield D, Singh N, Gupta R, Lambers E, Thal M, Mackie A, Hoxha E, Ramirez V, Qui G, Sadayappan S, Ghosh AK, Kishore R (2012) Interleukin-IL-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-κB. Circulation 126:418–449. https://doi.org/10.1161/CIRCULATIONAHA.112.112185

Waehre T, Halvorsen B, Damas JK (2002) Inflammatory imbalance between IL-10 and TNF-alpha in unstable angina potential plaque stabilizing effects of IL-10. Eur J Clin Invest 32:803–810. https://doi.org/10.1046/j.1365-2362.2002.01069.x

Walter MR (2002) Structure of interleukin-10/interleukin-10R1 complex: a paradigm for class 2 cytokine activation. Immunol Res 26:303–308. https://doi.org/10.1385/IR:26:1-3:303

Xiao X, Song BL (2013) SREBP: a novel therapeutic target. Acta Biochim Biophys Sin 45:2–10. https://doi.org/10.1093/abbs/gms112

Yang Z, Zingarelli B, Szabo C (2000) Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. Circulation 101:1019–1026. https://doi.org/10.1161/01.CIR.101.9.1019

Yao L, Huang K, Huang D, Wang J, Guo H, Liao Y (2008) Acute myocardial infarction induced increases in plasma tumor necrosis factor-alpha and interleukin-10 are associated with the activation of poly(ADP-ribose) polymerase of circulating mononuclear cell. Int J Cardiol 123:366–368. https://doi.org/10.1016/j.ijcard.2007.06.069

Yeang C et al (2019) Reduction of myocardial ischaemia–reperfusion injury by inactivating oxidized phospholipids. Cardiovasc Res 115:179–189. https://doi.org/10.1093/cvr/cvy136

Yndestad A, Damas JK, Oie E, Ueland T, Gullestad L, Aukrust P (2007) Role of inflammation in the progression of heart failure. Curr Cardiol Rep 9:236–241. https://doi.org/10.1007/BF02938356

Yu L, Feng Z (2018) The role of Toll-like receptor signaling in the progression of heart failure. Mediators Inflamm 2018:9874109. https://doi.org/10.1155/2018/9874109

Zhang W, Chancey AL, Tzeng HP, Zhou Z, Lavine KJ, Gao F, Sivasubramanian N, Barger PM, Mann DL (2011) The development of myocardial fibrosis in transgenic mice with targeted overexpression of tumor necrosis factor requires mast cell-fibroblast interactions. Circulation 124:2106–2116. https://doi.org/10.1161/CIRCULATIONAHA.111.052399

Zlobine I, Gopal K, Ussher JR (2016) Lipotoxicity in obesity and diabetes-related cardiac dysfunction. Biochim Biophys Acta 1860:1555–1568. https://doi.org/10.1016/j.bbalip.2016.02.011

Gene Therapy in Liver Disease: Challenges and Outcomes

14

Madhumita Premkumar and Virendra Singh

Abstract

Liver-directed gene therapy (LDGT) has been proposed as a means of treating many single-gene inherited disorders. LDGT has been used to create genetic pharmacological products that can be used for treatment of liver diseases and liver cancers, by inhibiting the expression of harmful proteins, delivering antisense RNAs, dominant negative proteins and ribozymes. Neoplastic diseases such as hepatocellular carcinomas can be managed by using gene editing to eliminate tumour cells selectively and sparing the healthy liver tissue. A special therapeutic use has been in the induction of specific immune response or immunotherapy against neoplastic cells in hepatocellular carcinoma (HCC) with mixed results. This chapter explains the modalities of gene therapy delivery, liver diseases that can be managed with LDGT, vectors to deliver LDGT and the impact of gene editing on diagnostics and therapeutics in liver diseases.

Keywords

Gene therapy · CRISPR/Cas9 · Zinc finger nuclease · Transcription activator-like effector nucleases

Abbreviations

adv	Adenovirus vector
CRISPR/Cas9	Clustered regularly interspaced palindromic repeats/caspase 9
HDR	Homology-directed repair

M. Premkumar · V. Singh (✉)
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_14

hiPSC	Human induced pluripotent stem cells
iPSCs	Induced pluripotent stem cells
KO	Knock out
KRAB	Krüppel-associated box epigenetic repressor motif
LGDT	Liver-directed gene therapy
LNP	Lipid nanoparticle
NHEJ	Non-homologous end joining
rAAV	Recombinant adenoviral vector
TALEN	Transcription activator-like effector nucleases
ZFN	Zinc finger nuclease

14.1 Targeted Gene Editing

LDGT is under research to treat many inherited disorders of metabolism (Trevisan et al. 2020). This can be done by delivering the editing nucleases, antisense RNAs etc. to the target cell in vivo using a variety of vectors or ex vivo by removing pluripotent stem cells, editing them in culture and re-injecting them (Finn et al. 2018). This LGDT has also been used in hepatocellular carcinoma (Gaj et al. 2013; Kim et al. 2016). The role of immunotherapy in management of neoplastic diseases has come to the fore in recent years (Duan and Lam 2013; Chen et al. 2014). Most rare inherited disorders of the liver are caused by gene defects including inherited mutations, loss of function of genes for protein expression for enzymes and proteins like ornithine transcarbamylase deficiency or alpha-1 antitrypsin deficiency. Such single-gene defects are good candidates for correction using traditional and new gene therapy strategies. The main approach to genetic therapy or LDGT is to ensure delivery of genetic material or editing tools to the hepatocytes.

The delivery of the corrected gene increases the level of expression of the defective protein or deficient enzyme, thus ameliorating the phenotypic expression of the gene disorder. Specific examples of such gene disorders are phenylketonuria (PKU) (Blau et al. 2010; Strisciuglio and Concolino 2014), urea cycle defects like ornithine transcarbamylase deficiency (Lichter-Konecki et al. 2013, 1993–2019), tyrosinemia (Overturf et al. 1996), arginase deficiency and haemophilia (Park et al. 2015). Figure 14.1 shows the milestones in the road to development of gene editing.

Liver-directed gene-editing technology allows us to treat cancer or specific-gene defects by altering the genome and correcting genetic mutations directly (Arad et al. 2005). The most common strategies include the use of editing tools like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), mega nucleases and clustered regularly interspaced short palindromic repeats/associated Caspase endonuclease or the CRISPR/Cas9 system (Villiger et al. 2018). The use of CRISPR/Cas9 has revolutionized the field of gene therapy and Dr. Jennifer Doudna and Dr. Emmanuelle Charpentier won the Nobel Prize in Chemistry in 2020 for this discovery. These are programmable nucleases or DNA cutting enzymes that can

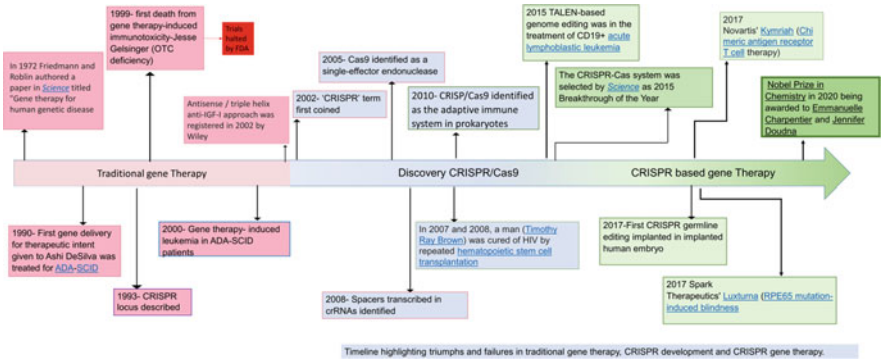


Fig. 14.1 Milestones in gene therapy research, triumphs and failures

target a specific DNA sequence to create a double strand break (DSB) at specific sites (Colella et al. 2017). The main techniques of gene editing are shown in Table 14.1. Once the dsDNA is cut, the cell detects the break as a lethal event and repairs the DNA and performs the repair through a homology-directed repair (HDR) or non-homologous end joining (NHEJ). The NHEJ repair requires native cellular ligases to re-join the cut ends of the cut DNA. This kind of DNA repair is error-prone and results in nucleotide insertions or deletions at the site of the break, enabling the intervention to be site-specific. These indels can result in knockout of the protein coded in the region through new frame-shift mutations, which can thus prevent transcription and expression of a deleterious gene (Gaj et al. 2013). HDR is often used to insert a desired DNA sequence as regions of homology with genomic DNA on either side of the cut ensuring homology of the arms. It is far easier to knock out a gene through NHEJ rather than introducing a new sequence through HDR (Stoddard 2011). A practical example of this process was the introduction of a CRISPR/Cas9 and cytidine deaminase with an RNA guide which can convert cytidine to uridine at a target site (Trevisan et al. 2017). Thus, Komor et al. created the first generation of cytosine base editors (BE1) (Komor et al. 2016). Cytosine base editors can introduce a cytosine to thymidine substitution with a DSB. The process of targeted repair can be enforced by adding a second enzyme or second-generation base editors (BE2) which inhibits base excision repair. This ensures that the edited gene is not corrected by the host cells' repair nucleases. BE3 further increases efficiency of this system by enhancing the cell-mediated correction of the non-edited strand. Since about 50% of pathological point mutations are c-G to T-A transitions, adenine base editors (ABE) were also created (Rees and Liu 2018).

On the other hand, TALENs and ZFNs rely on the catalytic site of the FokI restriction enzyme to cleave DNA (Gaudelli et al. 2017). Therefore ZFN and TALEN allow precise correction of genetic defects that result in disease or potentially activate tumour suppressor genes or inactivate oncogenes (Ho et al. 2018). One approach is to introduce genetic material like DMA of mRNA encoding the nuclease (ZFN, TALEN or CRISPR/Cas9 system) and a repair template which are delivered

Table 14.1 List of genome editing strategies

	CRISPR/Cas9	ZFN	TALEN	Meganucleases
Site of action of gene edit	RNA-DNA	Protein-DNA	Protein-DNA	Protein-DNA
Target sequence	Cas 9 cuts adjacent to protospacer adjacent motif (PAM), a short sequence of 2–6 base pairs varies among CRISPR/CAs9s from different species	Each zinc finger binds to a 3 bp DNA target. Assembly of 3–4 zinc finger modules is required for specificity in recognition	Each TALEN repeat binds a base pair of DNAs. Sequences targeted by TALEN effector repeats are typically directly preceded by a thymine (T) for HDR	The mega nuclease requires a preexisting recognition site
Off-target effects	CRISPR/cas 9 has highest off-target effects than TALENs, ZFNs and Mega nuclease	More potential off-target effects than TALENs	Least observed off-target effects than CRISPR/Cas9	Potential off-target effects
Multiplexing technology	CRISPR/Cas 9 is easiest	Technology is labour intensive	Technology is labour intensive	Technology is labour intensive
Application of editing tool	1. Using CRISPR for genome editing;	1. Genome editing	1. Identify and manipulate plant genomes	1. Genome editing especially creation of gene editing scaffolds
	2. CRISPR libraries for screening;	2. Genetic creation of disease models	2. Genetic manipulation to produce biofuels	
	3. CRISPR/cas9-mediated chromatin immunoprecipitation			
	4. Epigenetic editing 5. Live imaging of DNA/mRNA			
Applications in clinic	1. New therapeutic applications	Gene editing of CCR5 is autologous	Knock out gene models in cancer	1. *Hemoglobinopathies* 2. *Cystic fibrosis disease models*
		CD4+T cells of persons infected with HIV		
				–

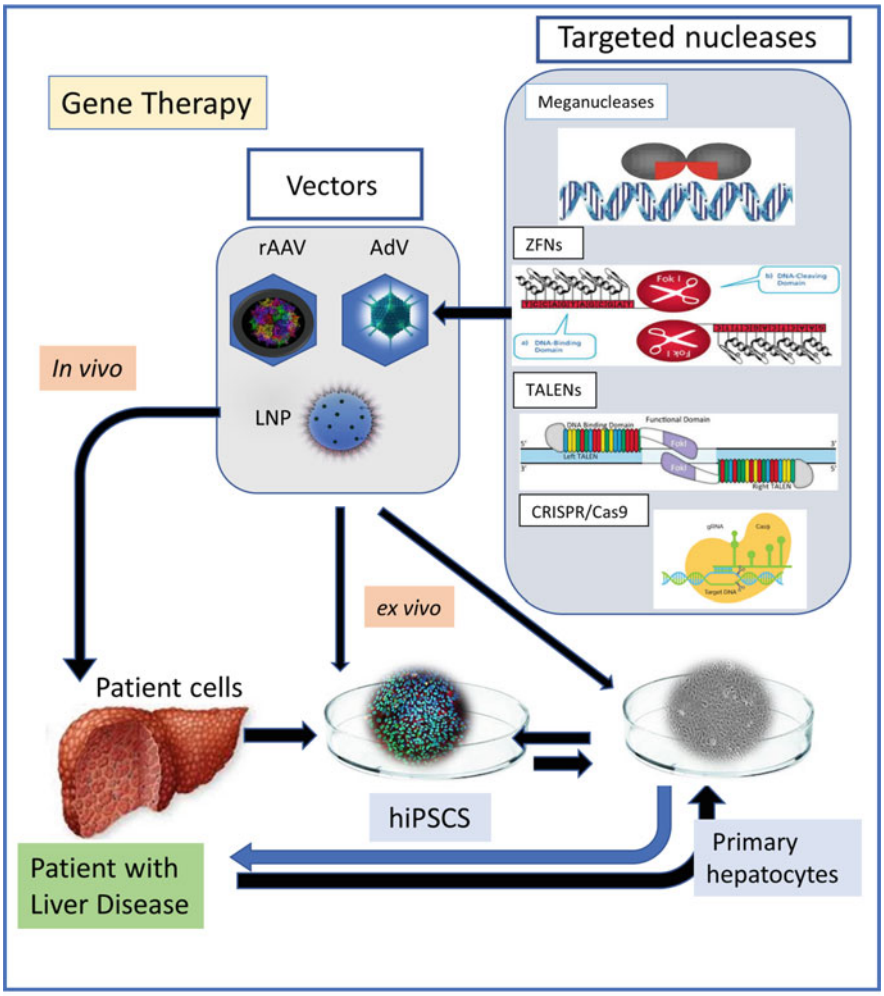
via the payload of vectors like viral vectors or lipid nanoparticles (LNPs), which can carry out the desired gene editing in vivo (Cong et al. 2013; Doudna and Charpentier 2014; Joung and Sander 2013). The mechanism of action of ZFN is that their zinc finger domains can bind to sites near the target area of DNA to be edited in the disease-related gene of interest. The ZFN then causes a DSB in the target DNA and a non-mutated sequence can be introduced to the cell via the payload of a vector and used as a template for cellular repair processes (Lieber 2010).

Another nuclease is the TALEN system which can introduce a DSB to edit the genome. Two TALENs can be used to target a pair of binding sites flanking 16 bp spacers. The left and right TALENS can recognize the top and bottom strands of the target sites, and the FokI endonuclease and TALEN DNA domain fuse. When the FokI dimerizes, it cuts out the DNA between the left and right TALEN binding sites leading to a precise excision of a DNA sequence (Jasin and Rothstein 2013).

CRISPR/Cas 9 uses a guide RNA with a part of the RNA sequence bound to the Cas9 protein and the other part having a 20 bp sequence that engages in a classic Watson–Crick base pairing in order to recognize and bind to pre-selected sites on the host genome (Helleday et al. 2007). Once bound to the specified DNA site, the two lobes of Cas-NUC and REC surround the DNA and position their respective nucleases RuvC and HNH and make a DSB in the DNA. Many groups have engineered the CRISPR/Cas9 system to alter its function. Relevant for in vivo genomic editing for HCC tissue are the Cas9 nickase enzymes (Dominguez et al. 2016). These are either RuvC or HNH endonuclease domains are mutated making the Cas9 cut only one strand of DNA. By using 2 such 'Cas9 nickases, which cut only one strand each, with guide RNA sequences at sufficient distances, it is possible to create strictly cut ends of DNA which can be ligated using ligases. This reduces off-target editing by 50 to 1500-fold (Ran et al. 2013). Figure 14.2 shows a schematic diagram of how the gene-editing tools can be used in vivo and ex vivo to target genetic diseases or liver cancer using the available gene-editing tools.

14.2 Vectors for Delivery of Gene Therapy

Once we have corrected the gene product, we also need a delivery mechanism to the liver or the target organ. The histological structure of the liver parenchyma can be categorized as parenchymal and non-parenchymal cells (Jacobs et al. 2012). Non-parenchymal cells include liver sinusoidal endothelial cells (LSECs), fat storing Ito cells, pit cells (natural killer cells) and Kupffer cells. The non-parenchymal cells make up the unique the fenestrated sinusoids, which sieves vectors and acts as a barrier for drug delivery to the liver parenchymal cells (Braet and Wisse 2002).The size of these fenestrae is about 107 nm in humans, 150 nm in Sprague Dawley rats, 141 mm in C57/BL/6 rats, and 124 nm in Dutch belt rats. The size must be taken into consideration when using viral vectors in different studies (Szilágyi and Berriman 1994). Therefore, viral vectors to deliver nucleic acids like adenoviral vector (150 nm), adeno-associated virus (AAV) which is 22 nm, herpes simplex virus is 180 nm. The AAV being only 22 nm thus is a great candidate for gene therapy but its



Abbreviations : rAAV, recombinant adeno viral vector; adv, adenovirus vector; LNP, lipid nanoparticle; ZFN, zinc finger nuclease; hiPSC, human induced pluripotent stem cells; TALEN, transcription activator like effector nucleases; CRISPR/Cas9, clustered regularly interspaced palindromic repeats/ caspase 9.

Fig. 14.2 Methods of delivering gene therapy. Abbreviations: *rAAV* recombinant adeno viral vector, *adv* adenovirus vector, *LNP* lipid nanoparticle, *ZFN* zinc finger nuclease, *hiPSC* human induced pluripotent stem cells, *TALEN* transcription activator like effector nucleases, *CRISPR/Cas9* clustered regularly interspaced palindromic repeats/caspase 9

size limits the amount of agent that can be given in each AAV vector. The liposomal vectors have a diameter of 50–1000 nm. Therefore, it is clear from the size constraints, the AAV vector is taken up by liver parenchymal cells while the

liposomal vectors and adeno vectors are taken up by LSECS and Kupffer cells (Banerjee 2001; Kim et al. 2016).

The dual supply of the liver and preferential supply of HCC by the arterial system suggests that gene therapy can be delivered to tumour cells of HCC by intra-arterial injection, a method that is used in locoregional therapy when the tumour radiation and chemotherapeutic agent is injected in the hepatic artery (Duan and Lam 2013).

There are several new virus candidates for viral vectors like herpes simplex virus, Newcastle disease virus, alphavirus, rhabdovirus, measles and picornaviruses. Viral vectors are useful for LDGT, and immunotherapy (Chen 2007; Tao et al. 2001; Fu et al. 2012).

Viruses can cause cell death by indirect means by viral replication and spread and not only by the genes conveyed by the vectors (Chen et al. 2017). The use of conditionally replicative adenoviruses (CRADs) has been used for their anti-tumour action (Lin et al. 2014). CRADs contain partial deletions of early replication genes or entire E1A and E1B genes under an HCC or cancer-specific promoter (Curiel 2000; Hallenbeck et al. 1999; Brand et al. 1998). Onyx-015, is a CRAD lacking E1B, which leads to p53 destruction, RNA export, and stopping host protein production, which specifically attacks tumour cells by replicating only in p53-deficient cells (O'Shea et al. 2005). Another conditionally replicative virus was a modified herpes virus T VEC, which received FDA approval for use in melanoma in 2015 (Pol et al. 2015).

14.3 Adeno-Associated Virus

AAV is the most established gene delivery vector for use in liver disease. The first successful clinical trials for haemophilia gene replacement used AAV. This vector proves to be an effective delivery for LDGT, evades the immune system, and improved transduction efficiency. However due to its small size gene inserts, with the packaging capacity of only 5 kb of DNA. There have been studies to improve this capacity to increase the capsid size, increase target gene uptake, enhance intracellular processing and decrease immune response. Major advantages of AAV vectors are their rare frequency of genome integration and low genotoxicity. There have been no reported cases of cancer caused by AAV so far in 270 published clinical trials, though immunogenicity remains a concern. RGD peptide into the AAV6 capsid, combined with site-directed mutagenesis to avoid undesirable binding, can increase transduction efficiency up to eightfold in human cancer cells, including liver cancer (Sayroo et al. 2016; Yuan et al. 2013).

14.4 Vaccinia Virus

The vaccinia virus is from Pox virus family, Poxviridae. The PexaVec, a vaccinia virus, is conditionally replicative in HCC. It is dependent on host cells for expression of thymidine kinases and engages host anti-tumour immunity by expression of a

transgene granulocyte-macrophage colony-stimulating factor (GM-CSF). PexaVec results in the lysis of hepatocellular carcinoma cells, induces host immune response and disruption of the HCC vasculature. A phase III RCT for PexaVec in combination with sorafenib started in 2015. Results were expected in December 2020, but the trial was stopped by the company Silla Jendue to lack of clinical effect. It may be possible that results are obtained in combination with immunotherapy (Breitbach et al. 2015).

14.5 Microvesicles, Exosomes and Virosomes

Exosomes are microvesicles that carry cell signalling molecules, lipids, proteins or nucleic acids and are used by tumour cells. Exosomes are about 30–100 nm in diameter and are an important means for cell signalling, tumour regulation and immune cell responses (Chen et al. 2019; Abudoureyimu et al. 2019). Exosomes are composed of a lipid membrane which enclose a content payload of nucleic acids, hormones, proteins and have adhesion and receptor proteins on their surface for selective uptake (Zhang et al. 2014). After release from their progenitor cells like tumour cells, immune cells etc., exosomes travel to the recipient cells, fuse with their membranes, and release their payload into the cytoplasm, delivering the communication signal (Mathiyalagan and Sahoo 2017). In addition, that exosomes may be endocytosed by the recipient cell, through clathrin-mediated or receptor-mediated endocytosis or phagocytosis. Specifically, in hepatocarcinogenesis, exosomes increase cancer replication and local spread, act as chemoresistance activators, promoting angiogenesis promoters and initiate epithelial mesenchymal transition (Yang et al. 2017).

Therefore, it appears the use of exosomes in cancer gene therapy is counterintuitive. Tumour-derived exosomes can be modified to activate dendritic cells which in turn create a tumour-specific immune response. Exosomes act as delivery vectors for small molecules like DNA, mi RNA and siRNA which target oncogenes. Such exosomes can register a tumour-specific response due to selective uptake, low immunogenicity, low toxicity and naturally protects RNA from degradation. Exosomes can also be used to package miRNA to HCC. Adipose tissue-derived mesenchymal stem cells were transfected with miRNA 122 to produce miR-122 carrying exosomes.

14.6 Gene Editing in Hepatocellular Carcinoma

Intra-tumoral injection is a means of avoiding an excessive immune response and is preferred for adenoviral delivery in HCC. It is possible to make the viral capsid and increase uptake in cancer cells by using polyethylene glycol or RGD, peptide that upregulates integrin. A PEGylated arginine-grafted bio reducible poly (CBA-DAH) polymer conjugated with hepatoma-targeting HCBP1 peptides (ABP-PEG-HCBP1) has been recently used to mask the surface of a GFP-expressing oncolytic virus for treatment of HCC (Fujiwara 2019). This increased the uptake in HCC and reduces

the innate immune response. The use of the human telomerase hTERT promoter to increase the expression of viral genes E1A and E1B for selective replication and lysis of cancer cells is another example of LDGT. The use of oncolytic virus OBP-301 (telomelysin) is potent and has cancer-selective anti-HCC activity in an immunocompetent mouse HCC model (NCT02293850 2014).

A study showed that intra-tumoral injection of a replication-deficient Ad carrying the HSV thymidine kinase (HSVtk) reported fever. About 60% of patients did have brief tumour stabilization. Efforts have been made to improve tumour specificity by using a trans-splicing ribozyme to target hTERT RNA and convert it to HSVtk which enhances anti-HCC action in the mouse model (Sangro et al. 2010). HSVtk therapy that is AFP driven in conjunction with ganciclovir enhances tumour activity in a mouse model. The HSVtk phosphorylates the prodrug ganciclovir and converts it into a potent inducer of apoptosis able to block DNA synthesis (Durymanov and Reineke 2018). Another initiator of apoptosis is the TNF-related apoptosis-inducing ligand (TRAIL), which selectively acts on HCC cells with little effect on normal liver parenchyma (Liu et al. 2018). When TRAIL is combined with the cytokine IL-12, which, when delivered by oncolytic Ad, shows potent anti-tumour effects in an HCC mouse model (Zamboni et al. 2017). TRAIL and inhibitor of growth 4 (ING 4) also show anti-tumour activity in an HCC mouse model.

14.7 Clinical Approaches to Gene Therapy in Liver Disease

Editing the genome can be done either in vivo by delivering the editing enzymes to the parent cell via viral vectors, nanoparticles which contain the requisite gene-editing agents (CRISPR/Cas9, ZFNs or TALENS) with or without the donor template for in situ correction by directly injecting the patient. To this end, hepatocytes are collected from the patient, treated with the nucleases, and reinjected in the patient for ex-vivo gene editing. In addition, human pluripotent stem cells (hiPSCs) can be collected, modified with the use of the editing nucleases, differentiated into adult hepatocytes in vitro, and then these edited hepatocytes with the correct gene template can be transplanted back to the patient.

The classical diseases that are amenable to treatment and cure by LDGT include single-gene disorders like inborn errors of metabolism. The most common diseases currently being under evaluation for gene therapy are listed in Table 14.2.

14.8 Phenylketonuria

One of the first inborn errors of metabolism to be targeted via LDGT is phenylketonuria (PKU). This disease has autosomal recessive (AR) inheritance, and patients have mutant PAH mutations on both alleles. The disease presents with delayed neurological development, childhood seizures, musty smell, light skin and autism. However, gene editing of any one allele suffices to raise enough enzyme activity to ameliorate the symptoms of PKU. More than 50% are missense mutations and the

Table 14.2 Genome editing strategies applied to rare liver diseases

Disease State	Prevalence	Inheritance	Defective enzyme	Target gene	OMIM	Clinical phenotype	Clinical management	Gene editing approaches	Strategy for gene editing	Model
Hemophilia A (HA)/ hemophilia B (HB)	1 in 5000 males/1 in 30,000 males	XLR/XLR	Factor VIII for hemophilia A, factor IX for hemophilia B and factor XI for hemophilia C	F8/F9	306,700/ 306,900	Deep bleeding into joints, skin, surgical bleeding	Infusions of rFVIII or r fIX as the case may be	Promoter less rAAV; CRISPR/ Cas9; ZFNs; TALENs	Inversion flip-flop; HDR; targeted insertion in safe harbors	Patient derives hiPSCs; HA/CD4 null mice; HB mice; neonatal and adult hF9/HB mice; R333Q hemophilia mice model
α-1 antitrypsin deficiency (AATD)	1:5000–1: 7000 In North America. 1: 2500 in Europe. It is uncommon in Asians	Autosomal recessive	Alpha-1 antitrypsin	SERPINA1	613,490	Lung disease or liver disease, cirrhosis or emphysema	Intravenous injections of A1AT protein Lung disease, bronchodilator, lung transplant liver-transplant	ZFN-piggyBac transposon CRISPR/Cas9; promoter lessrAAV	HDR; NHEJ	iPSCs from patients PiZ mouse; C57BL/6J mouse
Phenylketonuria (PKU)	1:10,000–1:15,000	Autosomal recessive	Phenylalanine hydroxylase	PAH	261,600	Delayed development, impaired cognition, seizures, musty smell, light skin autism, motor, deficits, eczema	Diet changes, saproterin dihydrochloride	FokI-dCas9 system CRISPR-Cas9 gene editing	HDR; base editing	COS-7 cells; Pahenu adult mouse

Ornithine transcar bamylase deficiency	1:70,000	X-linked recessive	Ornithine transcarbamylase	OTC	311,250	Vomiting, headache, ataxia, hepatic encephalopathy, developmental disorders, mental retardation	Low protein diet, nitrogen scavenging measures Only proven therapy is liver transplant	CRISPR/Cas9	HDR	Spfash mouse
Argininemia	1: 1,100,000	Autosomal recessive	Arginase-1	ARG1	207,800	Hypotonia, microcephaly, slowing of growth, spasticity, loss of developmental milestones	Protein restriction	CRISPR/Cas9; TALENs	HDR	Arg-1 deficient mouse
Tyrosinemia type 1 (HTI)	1/100,000 births	Autosomal recessive	Fumarylacetoacetate hydrolase	FAH/HPD	276,700	Renal disease, liver failure, rickets, neurological crises, risk of hepatocellular carcinoma	Diet low in tyrosine and phenylalanine	CRISPR-Cas associated gene editors Nme-Cas9; Cas9 nickases; Promoterless rAAV;	HDR; microhomology mediatedend joining targeted sequence substitution; allelic exchange/ NHEJ; base editing	h −/− mice; fah −/− rats; Fahneo/PM mice; fah −/− primary hepatocytes; in utero

(continued)

Table 14.2 (continued)

Disease State	Prevalence	Inheritance	Defective enzyme	Target gene	OMIM	Clinical phenotype	Clinical management	Gene editing approaches	Strategy for gene editing	Model
Mucopolysaccharidoses (Hurler syndrome; Scheie syndrome; Hunter syndrome)	MPS1: 1: 100,000; MPS2: 1:100,000 males	X-linked recessive	Lysosomal enzyme defect	IDUA; IDS	607,014, 607,015, 607,016 (MPS1); 309,900 (MPS2) AR	Bone and joint abnormalities due to glycosaminoglycan accumulation, lung disease, storage disorder with corneal clouding hepato splenomegaly. Neurocognitive impairment, cardiac abnormalities	Aldurazyme, enzyme replacement therapy. Cord blood transplant; bone marrow transplantation	ZFNs; CRISPR/ Cas9	Targeted insertion in safe harbors; HDR; allelic exchange	IDSy/-KO mice; fibroblasts from patients; iPSCs from idua KO mouse
Hyperchole-sterolemia	–	NA	LDL receptor gene, APO B gene, LDL RAP1 gene	PSK9	NA	High level of circulating concentration of low-density lipoprotein (LDL) cholesterol	Avoiding trans fats, use of statins, LDL apheresis	ZFN; meganuclease; CRISPR/Cas9: spCas9, Nme2Cas9 Gene editors dCas9-KRAB	NHEJ; transcription silencing; also, base editing-mediated gene silencing	Wild-type mice; FRG KO humanized mice; C57Bl/6 mice; macaques; in utero

Abbreviations: *CRISPR/Cas9* clustered regularly interspaced short palindromic repeat/(CRISPR)-associated nucleases 9, *spCas9* Streptococcus pyogenes Cas9, *saCas9* Staphylococcus aureus Cas9, *NmeCas9* Neisseria meningitides, *ZFNs* zinc finger nucleases, *TALENs* Transcription activator-like effector nucleases, Cas9, *KO* knock out, *KRAB* Krüppel-associated box epigenetic repressor motif, *NHEJ* non-homologous end joining, *HDR* homology-directed repair, *iPSCs* induced pluripotent stem cells

most severe mutation is c.1222C > T (p. Arg408Trp), which is a single nucleotide variant, amenable to correction. The most common genotype is p. [Arg408Trp]; [Arg408Trp] (Blau et al. 2014). Pan et al. reported the first gene-editing approach aimed to correct the c.1222C > T variant. One of the approaches to correct PKU involves a CRISPR/Cas9 system with a deactivated Cas9 enzyme and an RNA-guided FoKI nuclease to repair the defect in vitro (Pan et al. 2016).

14.9 Ornithine Transcarbamylase Deficiency

Ornithine transcarbamylase deficiency (OTC) is an X-linked IEM and a common urea cycle defect. Pathogenic variants in the OTC gene results in reduced activity of OTC enzyme causing hyperammonaemia, respiratory alkalosis, and hepatic encephalopathy. Sometimes liver transplantation is required in case of hyperammonaemic crises. OTC deficiency can be corrected using a CRISPR/Cas9 nuclease in a mouse model with a partial deficiency (spf *ash* mouse) (Hodges and Rosenberg 1989). This single nucleotide defect, with a G > A mutation at the splice site of exon 4, results in aberrant mRNA splicing and reduced level of OTC mRNA and protein. The genetic material is delivered by an rAAV system (Yang et al. 2016).

14.10 Argininemia

Arginase-1 deficiency is another AR IEM that involves a critical step in the urea cycle, affecting the hydrolysis of arginine to urea and ornithine. ARG1 gene mutations result in deficiency of this enzyme and results in a clinical syndrome with hepatic encephalopathy. The condition is managed by severe restriction of proteins in diet. Enzyme replacement has been attempted with limited success (Diez-Fernandez et al. 2018). Sin et al. created an inducible mouse model via Cre-mediated excision of exons 7 and 8 of Arg1 gene and tried to correct gene defect by a CRISPR/Cas9 system associated with a piggyBac technology in induced pluripotent stem cells which can be differentiated into hepatocytes and macrophages (Sin et al. 2013). Other mouse models have been described. Although iPSCs could be treated with gene repair but did not recover complete urea cycle function (Sin et al. 2017). On the other hand, iPSCs differentiated in macrophages could develop sufficient arginase-1 expression (Sin et al. 2018).

14.11 Alpha 1 Antitrypsin Deficiency

Alpha-1 antitrypsin (AAT) deficiency is an AR disease caused by defects in both alleles ofSERPINA1, the gene which encodes the protein alpha-1antitrypsin. AAT's deficiency results in a phenotype with chronic obstructive pulmonary disease or emphysema and in hepatic phenotype as cirrhosis. The commonest mutation in *SERPINA1* is c.1096G > A, which causes a p. Glu342Lys substitution, that results

in the production of the Z variant of AAT (Stoller et al. 2006, 1993–2019). Yusa et al. provided a proof of concept gene therapy using hiPSCs from an AATD patient and a combination piggyBac transposons and a ZFN to create a homozygous correction of the c.1096G > A *SERPINA1* point mutation (Yusa et al. 2011).

14.12 Hereditary Tyrosinemia

Hereditary tyrosinemia (HT1) in an IEM affecting tyrosine catabolism due to impaired function of fumarylacetoacetate hydrolase (FAH), which is an enzyme that catalyses the final step of fumarylacetoacetate metabolism (Paulk et al. 2010). Mutations in the AR HT1 is caused by biallelic mutation, and result in accumulation of two end products of tyrosine metabolism, i.e., fumaryl and maleyl acetoacetate which leads to hepatic dysfunction and renal tubular damage (Junge et al. 2018). Yin et al. presented a model for correction of the gene defect with a CRISPR-based system causing HDR of the defect. In this model, a vector with payload co-expressing sg RNA and Cas9 was created and the tail vein was injected in fah −/− mice. The ss DNA homology sequence was done, and the functional deficiency of FAH was restored reversing the liver damage (Yin et al. 2014, 2016).

14.13 Haemophilia

Haemophilia is an important X-linked inherited bleeding disorder which famously affected several royals of European descent, and clinically presents as bleeding into large joints and other sites of internal bleeding. Although several genetic defects result in this phenotype, the most common mutation is in the F8 gene resulting in haemophilia type A. the F8 gene mutation results in deficiency of coagulation factor VIII and the F9 gene mutation results in haemophilia B with deficiency of factor IX. Haemophilia A is one of the most common genetic bleeding disorders, and multiple mutations result in the same phenotype (Naylor et al. 1993). The severity of the defect and % factor activity results in an increasing disease severity (Bagnall et al. 2002).The genetic defect in more than half the cases of haemophilia type A is due to two types of chromosomal inversions that result from non-allelic HDR in intron 1 or 22 and homologous regions upstream of the F8 gene. CRISPR/Cas9 editing tools or TALENS were used to correct the mutations by doing a flip flop of these homologous region inversion mutations in hiPSCs (Park et al. 2014; Li et al. 2011). Sangamo Therapeutics reported the successful repair of the gene defect in a haemophilic patient in December 2018 (Clinical Trial: NCT02695160). This method used a ZFN genome editor delivered by rAAV using SB-FIX which introduced the corrective copy of the Factor IX cDNA into the Alb locus. The treatment of haemophilia using gene therapy is a cornerstone of successful therapy (Lyu et al. 2018; Sung et al. 2019; Stephens et al. 2019).

14.14 Hypercholesterolemia

This genetic disorder results in high levels of serum cholesterol predisposing the patient to coronary artery disease (CAD), stroke and atherosclerosis. Statins are used to reduce serum cholesterol, but many patients do not tolerate statins or develop transaminitis. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a therapeutic LDGT (Ding et al. 2014; Wang et al. 2016). The PSCSK9 works as a low-density lipoprotein (LDL) receptor blocker, wherein a gain of function gene mutation causes high levels of LDL cholesterol leading to metabolic defects. This predisposes to premature cardiac and stroke risk. Conversely, persons with loss of function mutations have protection from cardiac risk. Yin et al. presented a model for treatment of hypercholesterolemia. This uses a spCas9 approach (in form of mRNA), with a chemically modified sgRNAs, termed enhanced sgRNA delivered by LNPs injected intravenously in a mouse model. The group reported 83% editing efficiency using this method, with reduction of plasma mpcsk9 and cholesterol levels without any liver toxicity (Yin et al. 2017).

In conclusion, the era of gene therapy offers new and pragmatic cures for once debilitating liver diseases. With improvement of editing techniques and delivery vectors, it is possible to correct many defects either in vitro or in vivo. It is only a matter of time before the first methods of LDGT are made commercially available. The liver with its dual supply and its fenestrated epithelium is an ideal target of gene therapy. With the first in vivo ZFNs-based clinical trials for HB and MPS already on the run, genome editing. Intra-tumoral injection of gene-editing payloads is a useful means of managing hepatocellular carcinoma.

Conflict of Interest Neither author has any relevant conflict of interest to declare.

Source of Funding Nil.

References

Abudoureyimu M, Zhou H, Zhi Y, Wang T, Feng B, Wang R, Chu X (2019) Recent progress in the emerging role of exosome in hepatocellular carcinoma. Cell Prolif 52(2):e12541. https://doi.org/10.1111/cpr.12541. Epub 2018 Nov 5

Arad U, Zeira E, El-Latif MA, Mukherjee S, Mitchell L, Pappo O, Galun E, Oppenheim A (2005) Liver-targeted gene therapy by SV40-based vectors using the hydrodynamic injection method. Hum Gene Ther 16(3):361–371. https://doi.org/10.1089/hum.2005.16.361

Bagnall RD, Waseem N, Green PM, Giannelli F (2002) Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood 99(1):168–174. https://doi.org/10.1182/blood.v99.1.168

Banerjee R (2001) Liposomes: applications in medicine. J Biomater Appl 16(1):3–21. https://doi.org/10.1106/RA7U-1V9C-RV7C-8QXL

Blau N, van Spronsen FJ, Levy HL (2010) Phenylketonuria. Lancet 376(9750):1417–1427. https://doi.org/10.1016/S0140-6736(10)60961-0

Blau N, Shen N, Carducci C (2014) Molecular genetics and diagnosis of phenylketonuria: state of the art. Expert Rev Mol Diagn 14(6):655–671. https://doi.org/10.1586/14737159.2014.923760. Epub 2014 May 31

Braet F, Wisse E (2002) Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. Comp Hepatol 1(1):1. https://doi.org/10.1186/1476-5926-1-1

Brand K, Löser P, Arnold W, Bartels T, Strauss M (1998) Tumor cell-specific transgene expression prevents liver toxicity of the adeno-HSVtk/GCV approach. Gene Ther 5(10):1363–1371. https://doi.org/10.1038/sj.gt.3300728

Breitbach CJ, Bell JC, Hwang TH, Kirn DH, Burke J (2015) The emerging therapeutic potential of the oncolytic immunotherapeutic Pexa-Vec (JX-594). Oncolytic Virother 4:25–31. https://doi.org/10.2147/OV.S59640. eCollection 2015

Chen H (2007) Comparative observation of the recombinant adeno-associated virus 2 using transmission electron microscopy and atomic force microscopy. Microsc Microanal 13(5): 384–389. https://doi.org/10.1017/S1431927607070808

Chen S, Chen J, Xi W, Xu W, Yin G (2014) Clinical therapeutic effect and biological monitoring of p53 gene in advanced hepatocellular carcinoma. Am J Clin Oncol 37(1):24–29. https://doi.org/10.1097/COC.0b013e3181fe4688

Chen A, Zhang Y, Meng G, Jiang D, Zhang H, Zheng M, Xia M, Jiang A, Wu J, Beltinger C, Wei J (2017) Oncolytic measles virus enhances antitumour responses of adoptive $CD8^{+}NKG2D^{+}$ cells in hepatocellular carcinoma treatment. Sci Rep 7(1):5170. https://doi.org/10.1038/s41598-017-05500-z

Chen R, Xu X, Tao Y, Qian Z, Yu Y (2019) Exosomes in hepatocellular carcinoma: a new horizon. Cell Commun Signal 17(1):1. https://doi.org/10.1186/s12964-018-0315-1

Colella P, Ronzitti G, Mingozzi F (2017) Emerging issues in AAV-mediated in vivo gene therapy. Mol Ther Methods Clin Dev 8:87–104. https://doi.org/10.1016/j.omtm.2017.11.007. eCollection 2018 Mar

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121): 819–823. https://doi.org/10.1126/science.1231143. Epub 2013 Jan 3

Curiel DT (2000) The development of conditionally replicative adenoviruses for cancer therapy. Clin Cancer Res 6(9):3395–3399

Diez-Fernandez C, Rüfenacht V, Gemperle C, Fingerhut R, Häberle J (2018) Mutations and common variants in the human arginase 1 (ARG1) gene: Impact on patients, diagnostics, and protein structure considerations. Hum Mutat 39(8):1029–1050. https://doi.org/10.1002/humu.23545. Epub 2018 Jun 21

Ding Q, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN, Cowan CA, Rader DJ, Musunuru K (2014) Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. Circ Res 115(5):488–492. https://doi.org/10.1161/CIRCRESAHA.115.304351. Epub 2014 Jun 10

Dominguez AA, Lim WA, Qi LS (2016) Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. Nat Rev Mol Cell Biol 17(1):5–15. https://doi.org/10.1038/nrm.2015.2. Epub 2015 Dec 16

Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science (New York, NY) 346(6213):1258096. https://doi.org/10.1126/science.1258096

Duan F, Lam MGEH (2013) Delivery approaches of gene therapy in hepatocellular carcinoma. Anticancer Res 33:4711–4718

Durymanov M, Reineke J (2018) Non-viral delivery of nucleic acids: insight into mechanisms of overcoming intracellular barriers. Front Pharmacol 9:971. https://doi.org/10.3389/fphar.2018.00971. eCollection 2018

Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J, Shah RR, Shah A, Ling D, Growe J, Pink M, Rohde E, Wood KM, Salomon WE, Harrington WF, Dombrowski C, Strapps WR, Chang Y, Morrissey DV (2018) A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. Cell Rep 22(9):2227–2235. https://doi.org/10.1016/j.celrep.2018.02.014

Fu X, Rivera A, Tao L, De Geest B, Zhang X (2012) Construction of an oncolytic herpes simplex virus that precisely targets hepatocellular carcinoma cells. Mol Ther 20(2):339–346. https://doi.org/10.1038/mt.2011.265. Epub 2011 Dec 6

Fujiwara T (2019) Multidisciplinary oncolytic virotherapy for gastrointestinal cancer. Ann Gastroenterol Surg 3(4):396–404. https://doi.org/10.1002/ags3.12270. eCollection 2019 Jul

Gaj T, Gersbach CA, Barbas CF 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31(7):397–405. https://doi.org/10.1016/j.tibtech.2013.04.004. Epub 2013 May 9

Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature 551(7681):464–471. https://doi.org/10.1038/nature24644. Epub 2017 Oct 25

Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J, Phipps S, Chiang YL (1999) A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum Gene Ther 10(10):1721–1733. https://doi.org/10.1089/10430349950017725

Helleday T, Lo J, van Gent DC, Engelward BP (2007) DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair 6(7):923–935. https://doi.org/10.1016/j.dnarep.2007.02.006. Epub 2007 Mar 23

Ho BX, Loh S, Chan WK, Soh BS (2018) In vivo genome editing as a therapeutic approach. Int J Mol Sci 19(9):2721. https://doi.org/10.3390/ijms19092721

Hodges PE, Rosenberg LE (1989) The spfash mouse: a missense mutation in the ornithine transcarbamylase gene also causes aberrant mRNA splicing. Proc Natl Acad Sci U S A 86(11):4142–4146. https://doi.org/10.1073/pnas.86.11.4142

Jacobs F, Gordts SC, Muthuramu I, De Geest B (2012) The liver as a target organ for gene therapy: state of the art, challenges, and future perspectives. Pharmaceuticals (Basel, Switzerland) 5(12):1372–1392. https://doi.org/10.3390/ph5121372

Jasin M, Rothstein R (2013) Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol 5(11):a012740. https://doi.org/10.1101/cshperspect.a012740

Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14(1):49–55. https://doi.org/10.1038/nrm3486. Epub 2012 Nov 21

Junge N, Yuan Q, Vu TH, Krooss S, Bednarski C, Balakrishnan A, Cathomen T, Manns MP, Baumann U, Sharma AD, Ott M (2018) Homologous recombination mediates stable Fah gene integration and phenotypic correction in tyrosinaemia mouse-model. World J Hepatol 10(2):277–286. https://doi.org/10.4254/wjh.v10.i2.277

Kim KI, Chung HK, Park JH, Lee YJ, Kang JH (2016) Alpha-fetoprotein-targeted reporter gene expression imaging in hepatocellular carcinoma. World J Gastroenterol 22(27):6127–6134. https://doi.org/10.3748/wjg.v22.i27.6127

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533(7603):420–424. https://doi.org/10.1038/nature17946. Epub 2016 Apr 20

Li H, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA (2011) In vivo genome editing restores haemostasis in a mouse model of haemophilia. Nature 475(7355):217–221. https://doi.org/10.1038/nature10177

Lichter-Konecki U, Caldovic L, Morizono H, Simpson K (2013) In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A (eds) (Updated 2016) Ornithine Transcarbamylase Deficiency. GeneReviews. Review. https://www.ncbi.nlm.nih.gov/books/NBK154378/

Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem 79:181–211. https://doi.org/10.1146/annurev.biochem.052308.093131

Lin Y, Zhang H, Liang J, Li K, Zhu W, Fu L et al (2014) Identification and characterization of alphavirus M1 as a selective oncolytic virus targeting ZAP-defective human cancers. Proc Natl

Acad Sci U S A 111(42):E4504–E4512. https://doi.org/10.1073/pnas.1408759111. Epub 2014 Oct 6

Liu CH, Chern GJ, Hsu FF, Huang KW, Sung YC, Huang HC, Qiu JT, Wang SK, Lin CC, Wu CH, Wu HC, Liu JY, Chen Y (2018) A multifunctional nanocarrier for efficient TRAIL-based gene therapy against hepatocellular carcinoma with desmoplasia in mice. Hepatology 67(3):899–913. https://doi.org/10.1002/hep.29513. Epub 2018 Jan 29

Lyu C, Shen J, Wang R, Gu H, Zhang J, Xue F, Liu X, Liu W, Fu R, Zhang L, Li H, Zhang X, Cheng T, Yang R, Zhang L (2018) Targeted genome engineering in human induced pluripotent stem cells from patients with hemophilia B using the CRISPR-Cas9 system. Stem Cell Res Ther 9(1):92. https://doi.org/10.1186/s13287-018-0839-8

Mathiyalagan P, Sahoo S (2017) Exosomes-based gene therapy for microRNA delivery. Methods Mol Biol 1521:139–152. https://doi.org/10.1007/978-1-4939-6588-5_9

Naylor J, Brinke A, Hassock S, Green PM, Giannelli F (1993) Characteristic mRNA abnormality found in half the patients with severe haemophilia A is due to large DNA inversions. Hum Mol Genet 2(11):1773–1778. https://doi.org/10.1093/hmg/2.11.1773

NCT02293850 (2014) Phase I study to evaluate the safety and efficacy of telomelysin (OBP-301) in patients with hepatocellular carcinoma (2014). Clinical Trials.gov: https://clinicaltrials.gov/ct2/show/NCT02293850

O'Shea CC, Soria C, Bagus B, McCormick F (2005) Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. Cancer Cell 8(1):61–74. https://doi.org/10.1016/j.ccr.2005.06.009

Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou CN, Finegold M, Grompe M (1996) Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat Genet 12(3):266–273. https://doi.org/10.1038/ng0396-266

Pan Y, Shen N, Jung-Klawitter S, Betzen C, Hoffmann GF, Hoheisel JD, Blau N (2016) CRISPR RNA-guided FokI nucleases repair a PAH variant in a phenylketonuria model. Sci Rep 6:35794. https://doi.org/10.1038/srep35794

Park CY, Kim J, Kweon J, Son JS, Lee JS, Yoo JE, Cho SR, Kim JH, Kim JS, Kim DW (2014) Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs. Proc Natl Acad Sci U S A 111(25):9253–9258. https://doi.org/10.1073/pnas.1323941111. Epub 2014 Jun 9

Park CY, Kim DH, Son JS, Sung JJ, Lee J, Bae S, Kim JH, Kim DW, Kim JS (2015) Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. Cell Stem Cell 17(2):213–220. https://doi.org/10.1016/j.stem.2015.07.001. Epub 2015 Jul 23

Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA, Grompe M (2010) Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. Hepatology 51(4):1200–1208. https://doi.org/10.1002/hep.23481

Pol J, Kroemer G, Galluzzi L (2015) First oncolytic virus approved for melanoma immunotherapy. Oncoimmunology 5(1):e1115641. https://doi.org/10.1080/2162402X.2015.1115641. eCollection 2016

Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154(6):1380–1389. https://doi.org/10.1016/j.cell.2013.08.021. Epub 2013 Aug 29

Rees HA, Liu DR (2018) Base editing: precision chemistry on the genome and transcriptome of living cells. Nat Rev Genet 19(12):770–788. https://doi.org/10.1038/s41576-018-0059-1. Review

Sangro B, Mazzolini G, Ruiz M, Ruiz J, Quiroga J, Herrero I, Qian C, Benito A, Larrache J, Olagüe C, Boan J, Peñuelas I, Sádaba B, Prieto J (2010) A phase I clinical trial of thymidine kinase-based gene therapy in advanced hepatocellular carcinoma. Cancer Gene Ther 17(12):837–843. https://doi.org/10.1038/cgt.2010.40. Epub 2010 Aug 6

Sayroo R, Nolasco D, Yin Z, Colon-Cortes Y, Pandya M, Ling C, Aslanidi G (2016) Development of novel AAV serotype 6 based vectors with selective tropism for human cancer cells. Gene Ther 23(1):18–25. https://doi.org/10.1038/gt.2015.89. Epub 2015 Oct 8

Sin YY, Ballantyne LL, Mukherjee K, St Amand T, Kyriakopoulou L, Schulze A, Funk CD (2013) Inducible arginase 1 deficiency in mice leads to hyperargininemia and altered amino acid metabolism. PLoS One 8(11):e80001. https://doi.org/10.1371/journal.pone.0080001. eCollection 2013

Sin YY, Price PR, Ballantyne LL, Funk CD (2017) Proof-of-concept gene editing for the murine model of inducible arginase-1 deficiency. Sci Rep 7(1):2585. https://doi.org/10.1038/s41598-017-02927-2

Sin YY, Ballantyne LL, Richmond CR, Funk CD (2018) Transplantation of gene-edited hepatocyte-like cells modestly improves survival of arginase-1-deficient mice. Mol Ther Nucleic acids 10:122–130. https://doi.org/10.1016/j.omtn.2017.11.012. Epub 2017 Dec 1

Stephens CJ, Lauron EJ, Kashentseva E, Lu ZH, Yokoyama WM, Curiel DT (2019) Long-term correction of hemophilia B using adenoviral delivery of CRISPR/Cas9. J Control Release 298: 128–141. https://doi.org/10.1016/j.jconrel.2019.02.009. Epub 2019 Feb 13

Stoddard BL (2011) Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. Structure 19(1):7–15. https://doi.org/10.1016/j.str.2010.12.003

Stoller JK, Hupertz V, Aboussouan LS (2006) In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A (eds) (Updated 2020) Alpha-1 antitrypsin deficiency. GeneReviews. Review. https://www.ncbi.nlm.nih.gov/books/NBK1519/

Strisciuglio P, Concolino D (2014) New strategies for the treatment of phenylketonuria (PKU). Metabolites 4(4):1007–1017. https://doi.org/10.3390/metabo4041007

Sung JJ, Park CY, Leem JW, Cho MS, Kim DW (2019) Restoration of FVIII expression by targeted gene insertion in the FVIII locus in hemophilia A patient-derived iPSCs. Exp Mol Med 51(4): 1–9. https://doi.org/10.1038/s12276-019-0243-1

Szilágyi JF, Berriman J (1994) Herpes simplex virus L particles contain spherical membrane-enclosed inclusion vesicles. J Gen Virol 75(7):1749–1753. https://doi.org/10.1099/0022-1317-75-7-1749

Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, Barsoum J, Fawell SE (2001) Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. Mol Ther 3(1):28–35. https://doi.org/10.1006/mthe.2000.0227

Trevisan M, Palù G, Barzon L (2017) Genome editing technologies to fight infectious diseases. Expert Rev Anti-Infect Ther 15(11):1001–1013. https://doi.org/10.1080/14787210.2017.1400379. Epub 2017 Nov 8

Trevisan M, Masi G, Palù G (2020) Genome editing technologies to treat rare liver diseases. Transl Gastroenterol Hepatol 5:23. https://doi.org/10.21037/tgh.2019.10.10. eCollection 2020

Villiger L, Grisch-Chan HM, Lindsay H, Ringnalda F, Pogliano CB, Allegri G, Fingerhut R, Häberle J, Matos J, Robinson MD, Thöny B, Schwank G (2018) Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. Nat Med 24(10):1519–1525. https://doi.org/10.1038/s41591-018-0209-1. Epub 2018 Oct 8

Wang X, Raghavan A, Chen T, Qiao L, Zhang Y, Ding Q, Musunuru K (2016) CRISPR-Cas9 targeting of PCSK9 in human hepatocytes *in vivo*-brief report. Arterioscler Thromb Vasc Biol 36(5):783–786. https://doi.org/10.1161/ATVBAHA.116.307227. Epub 2016 Mar 3

Yang Y, Wang L, Bell P, McMenamin D, He Z, White J, Yu H, Xu C, Morizono H, Musunuru K, Batshaw ML, Wilson JM (2016) A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. Nat Biotechnol 34(3):334–338. https://doi.org/10.1038/nbt.3469. Epub 2016 Feb 1

Yang N, Li S, Li G, Zhang S, Tang X, Ni S, Jian X, Xu C, Zhu J, Lu M (2017) The role of extracellular vesicles in mediating progression, metastasis and potential treatment of hepatocellular carcinoma. Oncotarget 8(2):3683–3695. https://doi.org/10.18632/oncotarget.12465

Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T, Anderson DG (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and

phenotype. Nat Biotechnol 32(6):551–553. https://doi.org/10.1038/nbt.2884. Epub 2014 Mar 30

Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, Xue W, Anderson DG (2016) Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol 34(3): 328–333. https://doi.org/10.1038/nbt.3471. Epub 2016 Feb 1

Yin H, Song CQ, Suresh S, Wu Q, Walsh S, Rhym LH, Mintzer E, Bolukbasi MF, Zhu LJ, Kauffma K, Mou H, Oberholzer A, Ding J, Kwan SY, Bogorad RL, Zatsepin T, Koteliansky V, Wolfe SA, Xue W, Langer R, Anderson DG (2017) Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. Nat Biotechnol 35(12):1179–1187. https://doi.org/10.1038/nbt.4005. Epub 2017 Nov 13

Yuan L, Zhao H, Zhang L, Liu X (2013) The efficacy of combination therapy using adeno-associated virus-mediated co-expression of apoptin and interleukin-24 on hepatocellular carcinoma. Tumour Biol 34(5):3027–3034. https://doi.org/10.1007/s13277-013-0867-z. Epub 2013 Aug 2

Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE, Miranda E, Ordóñez A, Hannan NR, Rouhani FJ, Darche S, Alexander G, Marciniak SJ, Fusaki N, Hasegawa M, Holmes MC, Di Santo JP, Lomas DA, Bradley A, Vallier L (2011) Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478(7369):391–394. https://doi.org/10.1038/nature10424

Zamboni CG, Kozielski KL, Vaughan HJ, Nakata MM, Kim J, Higgins LJ, Pomper MG, Green JJ (2017) Polymeric nanoparticles as cancer-specific DNA delivery vectors to human hepatocellular carcinoma. J Control Release 263:18–28. https://doi.org/10.1016/j.jconrel.2017.03.384. Epub 2017 Mar 27

Zhang Y, Li L, Yu J, Zhu D, Zhang Y, Li X, Gu H, Zhang CY, Zen K (2014) Microvesicle-mediated delivery of transforming growth factor β1 siRNA for the suppression of tumor growth in mice. Biomaterials 35(14):4390–4400. https://doi.org/10.1016/j.biomaterials.2014.02.003. Epub 2014 Feb 22

Interrupting Crystal to Calculus Conversion: The Future of Research in Urolithiasis

15

Sudheer Kumar Devana, Aditya Prakash Sharma, and Kapil Chaudhary

Abstract

Urolithiasis is one of the most important diseases in urology accounting for a significant health burden across the world. There has been a significant advancement in the endourological management of urolithiasis owing to improved optics, innovation in flexible ureteroscopy and stone ablation technologies as well. However, the progress of preventive management of urolithiasis has not occurred at the same pace. Lot of basic and clinical research on preventive strategies has been performed during the past few decades with minimal translation into routine clinical practice. The exact pathophysiology of stone formation still remains elusive. Various mechanisms and hypotheses for stone formation have been described. The present writeup is an attempt to review these proposed mechanisms and strategies to interrupt crystal to calculus formation with an emphasis on potential areas of future research.

Keywords

Urolithiasis · Pathogenesis · Future · Research · Nucleation · Randall plaque · Oxidative stress · Crystal growth · Aggregation · Supersaturation

15.1 Introduction

Evaluations of trends in the prevalence of urolithiasis across the globe have shown that there has been a steady increase in the burden of renal stone disease in most countries over the past three decades (Raheem et al. 2017). This could be attributed

S. K. Devana (✉) · A. P. Sharma · K. Chaudhary
Department of Urology, Post graduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_15

to various reasons like sedentary lifestyle, eating habits and even global warming. Renal stones can be broadly categorized into two types viz. calcium-containing and non-calcium-containing stones. Calcium-containing stones are the most common type which includes calcium oxalate (60%), hydroxyapatite (20%) and brushite stones (2%). The non-calcium-containing stones include uric acid (7%), struvite (7%), cysteine stones (1–3%) and others (Partin et al. 2020). The pathophysiology behind renal stone formation is multifactorial and has been attributed to dietary, environmental, genetic and various other causes. The mechanism of formation and preventive strategies for management of non-calcium stones like uric acid, struvite and cysteine stones have been described with clarity in the existing literature. Ironically the exact pathophysiology behind formation of the most common calcium-containing stones is still not clear.

15.2 Pathophysiology of Renal Stone Formation (Fig. 15.1)

Kok and Khan have described the free particle and fixed particle models of renal stone formation (Kok and Khan 1994). Free particle model suggests that stone formation occurs after intra-tubular precipitation and intra-tubular plug formation. Whereas, fixed particle model defines that stone formation occur after papillary plaque formation. Based on fixed particle model stone formation takes a very long time which is commonly seen in idiopathic renal stone formers. Whereas, the free

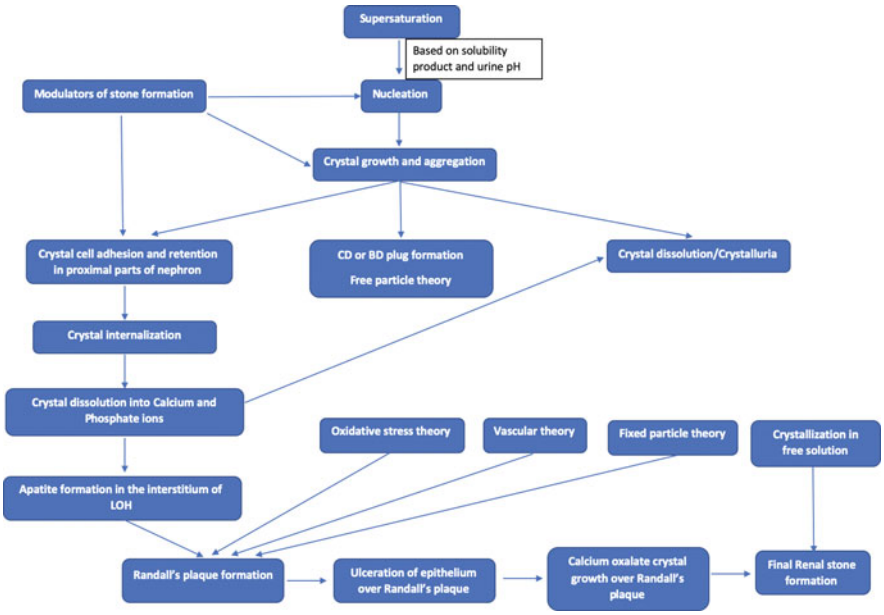


Fig. 15.1 Overview of various mechanisms of calcium containing renal stone formation. *CD* Collecting duct, *BD* Bellini duct, *LOH* Loop of Henle

particle model leads to fast recurrence of stones secondary to some metabolic abnormalities (Kok et al. 2017).

Evan et al. have proposed four different mechanisms of renal stone formation (Evan et al. 2015). These include

(a) Overgrowth of calcium oxalate stone on an interstitial plaque/Randall's plaque (RP): This is the most common mechanism seen in patients of idiopathic calcium oxalate stone formers or patients with hypercalciuria.
(b) Overgrowth of calcium oxalate or hydroxyapatite stone over Bellini duct (BD) plugs: This is seen commonly in brushite stone formers and also in patients with renal tubular acidosis and those with hyperparathyroidism.
(c) Formation of microliths in inner medullary collecting ducts: These are classically seen in patients of cystinuria.
(d) Crystallization in free solution: This is seen in cystinuria patients, following obesity bypass surgery and massive bowel resection.

Stone formation involves a complex series of steps involving physiochemical changes which occur as urine passes through the various parts of nephron. These steps include supersaturation of urine, nucleation, crystal growth, aggregation, retention, crystal cell adhesion, RP formation and final calculus formation and are described as follows.

15.2.1 Supersaturation

An aqueous solution containing salts is said to be saturated when the addition of extra salt after that point leads to precipitation. At this point, the dissolved and the crystalline components of the solution are in equilibrium. The state beyond this point, where the solutes are in excess than needed for them to stay in a soluble state is called the supersaturated state. Presence of inhibitors in urine prevents crystallization of solutes even in the supersaturated state and in this state, urine is considered to be metastable. After a threshold called the upper limit of metastability, crystallization will occur (Tiselius 1996). Supersaturation differs for various stone-forming salts based on the solubility product and is also pH sensitive. For example, hydroxyapatite is the only salt which can be supersaturated in all parts of nephron, whereas calcium oxalate gets supersaturated in the region of the collecting duct (Rodgers et al. 2011).

15.2.2 Nucleation

Nucleation is the initial step in renal stone formation. In a supersaturated solution the free atoms, ions or molecules adhere to each other resulting in formation of microscopic precipitates. This occurs as the overall free energy of this precipitate is lesser than that of the liquid solution (Ratkalkar and Kleinman 2011). Because of the high concentration of calcium and phosphate ions in the loop of Henle and also the high

pH (>6) of urine at this level, nucleation of calcium phosphate crystals occur. Asplin et al. in rat models have proved that thin descending loop of Henle creates a solid calcium phosphate state for formation of immature calcium phosphate moieties (Asplin et al. 1996). Hence nucleation leads to formation of crystals which occur predominantly in the proximal parts of the nephron.

Nucleation can be heterogenous or homogenous. Homogenous nucleation means similar stone-forming salts adhere to each other forming a microscopic crystal. This is uncommon and occurs in conditions like cystinuria leading to the formation of pure cysteine stones. Whereas, heterogenous nucleation is more common and involves adhesion of multiple stone-forming salts through another molecule or substance which acts as a binding agent leading to formation of a larger precipitate. For example, mucopolysaccharides acts as a binding agent between the stone-forming salts. Shredded epithelial cells, intra-luminal casts, red blood cells can also be involved in heterogenous nucleation. This nucleation process is controlled by a lot of proteins which act as promotors or inhibitors (Alelign and Petros 2018). At a particular pH these moieties act as a suitable substrate for heterogenous nucleation. For example, at a pH above 6 apatite nuclei stimulate the formation of calcium oxalate monohydrate crystals. This accounts for the presence of mixed stones such as calcium oxalate monohydrate with small amounts of apatite (Tiselius 2011).

15.2.3 Growth and Agglomeration/Aggregation

Crystal growth is a process where crystals get attached to each other or to a matrix-coated surface leading to formation of a small mass of stone. Crystal aggregation involves attachment of small masses of stone to form a larger stone. Crystallization depends on the thermodynamics and kinetics of supersaturated solution (Kok et al. 1990). It is important to note that supersaturation of urine and crystalluria can also be present in normal individuals and is not exclusive for renal stone formers. However, stone formers tend to excrete larger crystals in urine due to increased chance of crystal agglomeration compared to healthy adults who excrete smaller crystals in urine. Hence, crystal agglomeration is a critical step in stone formation. Kok et al. have shown that impaired inhibition of crystal agglomeration in recurrent stone formers was associated with increased frequency of stone recurrence (Kok et al. 1986).

15.2.4 Crystal Cell Adhesion and Retention

Crystal cell adhesion or interaction is another critical event in stone formation. Without this interaction, a stone crystal will not have enough time to grow within the lumen of nephron despite crystal growth and aggregation. Binding to the renal tubular epithelial cells leads to crystal retention and its growth. By the induction of ionic and hydrogen bonds, increased adhesive force leads to crystal-tubular

epithelial cell adhesion (Rabinovich et al. 2006). Calcium oxalate monohydrate crystals get adhered to the microvilli on the apical surface of tubular epithelium by binding to specific receptors. The crystals will subsequently get internalized into the epithelial cells by endocytosis (Lieske et al. 1994). These crystals can have either of the two fates: be digested by macrophages or lysosomes and cleared in urine or increase in size further. Macromolecules in urine like Tamm-Horsfall protein (THP), fibronectin, nephrocalcin, hyaluronic acid, sialic acid and monocyte chemoattractant protein 1 (MCP 1) inhibit crystal cell binding (Verkoelen et al. 2000). Experimental studies have shown that increased calcium in urine induces osteopontin (OPN) gene expression which in turn promotes adhesion of calcium oxalate crystals to epithelial cells. Anionic molecules like phospholipids on epithelial cell membranes also promote attachment of calcium oxalate monohydrate crystals to renal tubules (Sheng et al. 2005).

15.2.5 Randall Plaque (RP) Formation

Alexander Randall first described subepithelial calcium phosphate deposits in the inter-tubular spaces of renal papilla (Randall 1936). They are predominantly made up of calcium phosphate and calcium carbonate. RPs act as nidus for calcium oxalate stone formation as evidenced by the presence of renal calculi found attached to the tip of renal papilla. Calcium oxalate stones collected from stone formers have a concave-shaped facet or umbilication on the stone surface representing the presumed adhesive site of RP. Two types of plaques have been described. Papillary lesion type 1 is the most common, which includes calcium phosphate deposits in the interstitium. Papillary lesion type 2 includes intra-tubular calcium phosphate deposits in Bellini ducts (BD) which is less common. Formation of these two types of plaques depends on the type of underlying metabolic abnormality. RPs are primarily found surrounding the thin loops of Henle. Scanning electron microscope studies have shown that the initial site of origin of the RP is the basement membrane of loop of Henle from where it spreads through the interstitium to the region of collecting ducts and finally to the subepithelial location of the renal papilla (Daudon et al. 2015).

Proposed mechanism of RP formation (papillary lesion type 1) can be described as follows. Supersaturation due to increased calcium and phosphate in urine along with low diuresis and increased pH leads to formation of calcium phosphate crystals. Asplin et al. have showed that long loops of Henle are often supersaturated with calcium phosphate get precipitated there only (Asplin et al. 1996). Later because of crystal cell interaction these crystals get endocytosed into the tubular epithelial cells. Within the cytoplasm calcium phosphate crystals dissolute into calcium and phosphate ions (Lieske et al. 1997). These ions diffuse into the interstitium at the level of basement membrane of thin loop of Henle and again get supersaturated leading to formation of apatite (Evan et al. 2003). It is shown that basement membrane of loop of Henle is thick composed of collagen with abundant mucopolysaccharides which help in retention of these crystals. The apatite increases in size with alternating layers

of calcium phosphate and macromolecules like OPN. The plaque extends within the interstitium from the level of loop of Henle to the collecting ducts and finally the renal papillary subepithelial location (Evan et al. 2007). In papillary 1 lesion, when the plaque increases in size, probably due to mechanical effects, the surface epithelium gets denuded exposing the plaque to the supersaturated calyceal urine. Calcium oxalate crystals which are already in the calyceal urine get attached to the exposed plaque. Slowly over a period of time crystal aggregation will lead to formation of a large calcium oxalate stone. The initiation and growth of this calculus depends on various factors like increased solute load, low diuresis and pH of urine. When the calculus attains a significant size, it gets detached probably due to its own weight and shearing forces in the calyx. The detached site on the calculus from the RP is seen as a concave depression. Later on, over a period of time this site is completely covered all around by calcium oxalate deposits and finally, this gets buried in the core of the calculus. This type of stone formation is classically seen in idiopathic calcium oxalate stone formers or in those patients with calcium oxalates stones due to hypercalciuria or hyperoxaluria.

In case of type 2 papillary lesion, calcium phosphate crystals become large enough due to high pH and heterogenous agglomeration and get stuck in the collecting ducts. In collecting ducts compared to the proximal parts of nephron the ion activity product of calcium oxalate is high with low urinary pH. Low urinary pH leads to dissolution of calcium phosphate crystal into respective ions. Subsequently, these free calcium ions complex with oxalate leading to formation of calcium oxalate crystals. Risk of calcium oxalate precipitation is high if calcium phosphate crystals are trapped in the BD. Hence calcium phosphate crystals form the base for calcium oxalate stones (Tiselius 2013). These stones like the type 1 papillary lesion get attached to the papillary tip at the opening of the collecting ducts. This type of stone formation is classically seen in brushite stone formers.

Recently, vascular hypothesis has also been proposed for RP formation. As per this hypothesis, the relative hypoxia in the renal papilla coupled with hyperosmolar surrounding milieu and a turbulent blood flow in the vas recta leads to initiation of RP formation. This hypothesis suggests that the RP formation is similar to other pathogenic processes like coronary artery atherosclerotic plaque formation (Stoller et al. 2004; Taylor and Stoller 2015).

15.3 Modulators of Stone Formation

Nucleation, crystal growth, aggregation and crystal cell adhesion processes are modulated by various low and high molecular weight substances. They can be ions or macromolecules which can act at single or multiple stages of stone formation. They can be in solution state or embedded in the stones as a part of the non-crystalline or organic component which is called the matrix component of a stone. These modulators are predominantly inhibitory in nature but at times can act as promotors of crystallization as well. They are one of the important reasons as to why healthy adults with supersaturated urine don't form stones. These inhibitors

adsorb onto the crystal surface, prevent attachment of other ions and interfere with the crystal lattice leading to inhibition of crystal growth and agglomeration.

These modulators can be proteins, glycosaminoglycans (GAG) or lipids. Proteins like THP, nephrocalcin, OPN, urinary prothrombin fragment 1, inter α inhibitor, monocyte MCP-1, bikunin (BK) and GAGs like chondroitin sulphate, heparan sulphate and hyaluronic acid act as modulators. Lipids like phospholipids and glycolipids also play an important role as modulators in stone pathogenesis (Alelign and Petros 2018; Aggarwal et al. 2013; Marangella et al. 2004).

15.4 Role of Oxidative Stress in Stone Pathogenesis

There has been recent evidence pointing towards the role of oxidative stress in renal stone formation. Biomineralization due to increased reactive oxygen species (ROS) has been incriminated as the possible mechanism similar to atherosclerosis. ROS include free radicals, atoms or molecules with unpaired electron and their metabolites like superoxide anion, nitric oxide, hydroxyl ion and hydrogen peroxide. These ROS are involved in cellular signalling, cell injury and inflammation. They also induce changes in gene expression and signal transduction leading to pathological changes in the body (Dröge 2002; Kamata and Hirata 1999).

In vitro studies have shown that hyperoxaluria or hypercalcemia leads to reactive oxygen species production resulting in renal epithelial cell injury. Studies have shown that cell injury occurs predominantly in the proximal tubule of nephron with loss of epithelial brush border as evidenced by presence of proximal tubular enzymes in urine samples (Baggio et al. 1983; Boonla et al. 2007). ROS are produced predominantly by NADPH oxidase in kidney and also from mitochondria (Khan et al. 2011; Cao et al. 2004). Hyperoxaluria and calcium oxalate crystals deposition leads to activation of renin-angiotensin system due to upregulation of renin. Increased angiotensin II stimulates NADPH oxidase leading to ROS production via protein kinase C pathway. ROS leads to activation of P38 mitogen-activated protein kinase (p38MAPK) pathway which upregulates various transcription factors like nuclear factor κβ (NF κβ), activated protein-1(AP-1), Runt-related transcription factor-2 (RUNX-2) and Osterix. Subsequently, various macromolecules like OPN, BK, MCP-1, matrix Gla protein (MGP), collagen, bone morphogenic protein (BMP) and bone sialoprotein (BSP) are generated which will inhibit crystal nucleation, growth and aggregation (Khan 2014). Cell injury and inflammation resulting in ROS production enhance the collagen deposition which is an excellent nucleator of calcium phosphate. Finally, calcified RP will be formed.

Hyperoxaluria and calcium oxalate crystals cause mitochondrial damage by increasing the membrane permeability via neutral sphingomyelinase and phospholipase A2 enzymes. Increased ROS, cytochrome C and decreased glutathione within mitochondria cause the activation of apoptosis via increased caspase activity. This results in cell death and exposure of basement membrane of renal tubular cells to crystals (Fig. 15.2).

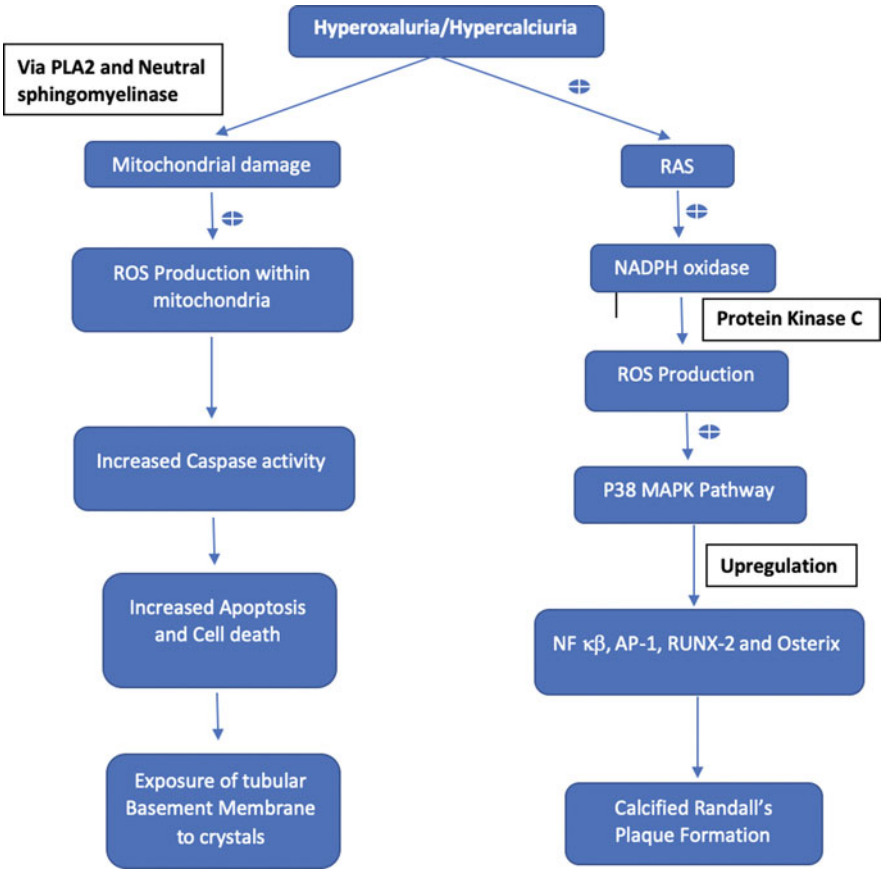


Fig. 15.2 Role of increased oxidative stress due to ROS production in the pathogenesis of renal stone formation. *RAS* Renin angiotensin system, *PLA2* Phospholipase A2, *ROS* Reactive oxygen species, *MAPK* Mitogen-activated protein kinase, *NF κβ* Nuclear factor κβ, *AP-1* Activated protein-1, *RUNX-2* Runt-related transcription factor-2

15.5 Interrupting Crystal to Calculus Conversion

Prevention of renal stone recurrence is the biggest challenge in the management of urolithiasis. Various experimental in vitro studies were performed at different stages of stone formation and have been summarized below.

15.5.1 Supersaturation

Avoiding supersaturation of urine is the cornerstone of most preventive approaches to stone recurrence. Maintaining high fluid intake is the most significant and

clinically proven preventive strategy for stone formation (Borghi et al. 1996; Cheungpasitporn et al. 2016). Modifiers of crystal growth and agglomeration cease to function in supersaturated urine, this being the premise for increased fluid intake advised in stone formers.

15.5.2 Crystal Nucleation

Phytate, Mg^{+2} and citrate are the modifiers which have been studied in inhibiting crystal nucleation. The studies on modulators of crystal nucleation are sparse and future studies focusing on this area are warranted. Increasing the upper limit of metastability holds the key to prevent crystallization.

15.5.3 Crystal Growth and Aggregation

This is the most extensively studied step so far in experimental trials for preventing stone recurrence. The most characteristic feature of modifiers is their high specificity to certain sites on the crystals. They are composed of carboxylic acid, sulphate, hydroxyl or phosphate groups which have specific affinity to crystal surface or free ions in urine. Citrate and hydroxy citrate are the best examples of modifiers in this category. The Carboxylic acid group binds to calcium and alcohol group to oxalate crystals by the formation of hydrogen bonds (Chung et al. 2016). This adsorption of modifiers on crystal surface will lead to step pinning, step blocking and inducing strain in crystal lattice (Olafson et al. 2016). Another mechanism of inhibiting crystal growth is by chemical modification of solute using L-cystine dimethyl ester in patients of cystine stones. This modification involves replacement of carboxylic acid with methyl ester group resulting in inhibition of crystal to crystal adhesion (Alamani and Rimer 2017). Citrate, magnesium and pyrophosphate shown to inhibit calcium phosphate crystal formation. Future research should be directed to creating macromolecules which have predominant inhibitory action on crystal aggregation.

15.5.4 Crystal Cell Integration

Change in crystal structure due to the above-mentioned modifiers of crystal growth or aggregation can also lead to decreased binding of crystal to anionic sites on tubular epithelium. Novel therapeutic agents with potential to alter the structure of the calcium oxalate crystals should be identified.

15.5.5 Reducing the Oxidative Stress

Experimental studies done on decreasing the oxidative stress in animal models have shown encouraging results in preventing stone formation.

(a) NADPH oxidase inhibition by apocynin has shown to decrease ROS and subsequently calcium oxalate deposition in hyperoxaluric rats (Zuo et al. 2011).
(b) Atorvastatin by decreasing the expression of $gp91^{phox}$ and $p22^{phox}$ subunits of NADPH oxidase has shown inhibition of crystal deposition in rats with hyperoxaluria (Tsujihata et al. 2008).
(c) Taurine by inhibiting the oxidative damage of ROS to mitochondria reduces crystal deposition (Li et al. 2009).
(d) *N*-acetyl cysteine has shown to inhibit an increase in cellular ceramides which are responsible for mitochondrial damage leading to apoptosis (Cao et al. 2000).
(e) Cyclosporine A treatment of hyperoxaluric rats also showed a decrease in mitochondrial damage and cell death (Niimi et al. 2012).

15.6 Conclusion

The exact mechanism of renal stone formation is still an enigma. Further research involving multiple steps/sites of crystal to calculus conversion can be explored in the near future for preventing stone recurrence. New research molecules should be discovered blocking the critical steps in calculus formation which can finally be translated into routine clinical use. Based on the type of renal calculus, new therapeutic agents should also be discovered for providing a complete comprehensive care to renal stone disease patients.

References

Aggarwal KP, Narula S, Kakkar M, Tandon C (2013) Nephrolithiasis: molecular mechanism of renal stone formation and the critical role played by modulators. Biomed Res Int 2013:292953. https://doi.org/10.1155/2013/292953

Alamani BG, Rimer JD (2017) Molecular modifiers of kidney stones. Curr Opin Nephrol Hypertens 26(4):256–265. https://doi.org/10.1097/MNH.0000000000000330

Alelign T, Petros B (2018) Kidney stone disease: an update on current concepts. Adv Urol 2018: 3068365. https://doi.org/10.1155/2018/3068365

Asplin JR, Mandel NS, Coe FL (1996) Evidence of calcium phosphate supersaturation in the loop of Henle. Am J Physiol 270(4 Pt 2):F604–F613. https://doi.org/10.1152/ajprenal.1996.270.4.F604

Baggio B, Gambaro G, Ossi E, Favaro S, Borsatti A (1983) Increased urinary excretion of renal enzymes in idiopathic calcium oxalate nephrolithiasis. J Urol 129(6):1161–1162. https://doi.org/10.1016/S0022-5347(17)52619-1

Boonla C, Wunsuwan R, Tungsanga K, Tosukhowong P (2007) Urinary 8-hydroxydeoxyguanosine is elevated in patients with nephrolithiasis. Urol Res 35(4):185–191. https://doi.org/10.1007/s00240-007-0098-0

Borghi L, Meschi T, Amato F, Briganti A, Novarini A, Giannini A (1996) Urinary volume, water and recurrences in idiopathic calcium nephrolithiasis: a 5-year randomized prospective study. J Urol 155(3):839–843

Cao LC, Honeyman T, Jonassen J, Scheid C (2000) Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells. Kidney Int 57(6):2403–2411. https://doi.org/10.1046/j.1523-1755.2000.00099.x

Cao LC, Honeyman TW, Cooney R, Kennington L, Scheid CR, Jonassen JA (2004) Mitochondrial dysfunction is a primary event in renal cell oxalate toxicity. Kidney Int 66(5):1890–1900. https://doi.org/10.1111/j.1523-1755.2004.00963.x

Cheungpasitporn W, Rossetti S, Friend K, Erickson SB, Lieske JC (2016) Treatment effect, adherence, and safety of high fluid intake for the prevention of incident and recurrent kidney stones: a systematic review and meta-analysis. J Nephrol 29(2):211–219. https://doi.org/10.1007/s40620-015-0210-4. Epub 2015 May 29

Chung J, Granja I, Taylor MG, Mpourmpakis G, Asplin JR, Rimer JD (2016) Molecular modifiers reveal a mechanism of pathological crystal growth inhibition. Nature 536(7617):446–450. https://doi.org/10.1038/nature19062. Epub 2016 Aug 17

Daudon M, Bazin D, Letavernier E (2015) Randall's plaque as the origin of calcium oxalate kidney stones. Urolithiasis 43(Suppl 1):5–11. https://doi.org/10.1007/s00240-014-0703-y. Epub 2014 Aug 7

Dröge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82(1): 47–95. https://doi.org/10.1152/physrev.00018.2001

Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao Y, Sommer AJ, Paterson RF, Kuo RL, Grynpas M (2003) Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. J Clin Invest 111(5):607–616. https://doi.org/10.1172/JCI17038

Evan AP, Coe FL, Lingeman JE, Shao Y, Sommer AJ, Bledsoe SB, Anderson JC, Worcester EM (2007) Mechanism of formation of human calcium oxalate renal stones on Randall's plaque. Anat Rec (Hoboken) 290(10):1315–1323. https://doi.org/10.1002/ar.20580

Evan AP, Worcester EM, Coe FL, Williams J Jr, Lingeman JE (2015) Mechanisms of human kidney stone formation. Urolithiasis 43((Suppl 1)(0 1)):19–32. https://doi.org/10.1007/s00240-014-0701-0. Epub 2014 Aug 10

Kamata H, Hirata H (1999) Redox regulation of cellular signaling. Cell Signal 11(1):1–14. https://doi.org/10.1016/s0898-6568(98)00037-0

Khan SR (2014) Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis. Transl Androl Urol 3(3):256–276. https://doi.org/10.3978/j.issn.2223-4683.2014.06.04

Khan SR, Khan A, Byer KJ (2011) Temporal changes in the expression of mRNA of NADPH oxidase subunits in renal epithelial cells exposed to oxalate or calcium oxalate crystals. Nephrol Dial Transplant 26(6):1778–1785. https://doi.org/10.1093/ndt/gfq692. Epub 2010 Nov 15

Kok DJ, Khan SR (1994) Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int. 46(3):847–854. https://doi.org/10.1038/ki.1994.341

Kok DJ, Papapoulos SE, Bijvoet OL (1986) Excessive crystal agglomeration with low citrate excretion in recurrent stone-formers. Lancet 1(8489):1056–1058. https://doi.org/10.1016/s0140-6736(86)91329-2

Kok DJ, Papapoulos SE, Bijvoet OL (1990) Crystal agglomeration is a major element in calcium oxalate urinary stone formation. Kidney Int 37(1):51–56. https://doi.org/10.1038/ki.1990.7

Kok DJ, Boellaard W, Ridwan Y, Levchenko VA (2017) Timelines of the "free-particle" and "fixed-particle" models of stone-formation: theoretical and experimental investigations. Urolithiasis 45(1):33–41. https://doi.org/10.1007/s00240-016-0946-x. Epub 2016 Dec 3

Li CY, Deng YL, Sun BH (2009) Taurine protected kidney from oxidative injury through mitochondrial-linked pathway in a rat model of nephrolithiasis. Urol Res 37(4):211–220. https://doi.org/10.1007/s00240-009-0197-1. Epub 2009 Jun 10

Lieske JC, Swift H, Martin T, Patterson B, Toback FG (1994) Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. Proc Natl Acad Sci U S A 91(15):6987–6991. https://doi.org/10.1073/pnas.91.15.6987

Lieske JC, Norris R, Swift H, Toback FG (1997) Adhesion, internalization and metabolism of calcium oxalate monohydrate crystals by renal epithelial cells. Kidney Int 52(5):1291–1301. https://doi.org/10.1038/ki.1997.454

Marangella M, Bagnis C, Bruno M, Vitale C, Petrarulo M, Ramello A (2004) Crystallization inhibitors in the pathophysiology and treatment of nephrolithiasis. Urol Int 72(Suppl 1):6–10. https://doi.org/10.1159/000076583

Niimi K, Yasui T, Hirose M, Hamamoto S, Itoh Y, Okada A, Kubota Y, Kojima Y, Tozawa K, Sasaki S, Hayashi Y, Kohri K (2012) Mitochondrial permeability transition pore opening induces the initial process of renal calcium crystallization. Free Radic Biol Med 52(7): 1207–1217. https://doi.org/10.1016/j.freeradbiomed.2012.01.005. Epub 2012 Jan 18

Olafson KN, Li R, Alamani BG, Rimer JD (2016) Engineering crystal modifiers: bridging classical and nonclassical crystallization. Chem Mater 28(23):8453–8465

Partin AW, Dmochowski RR, Kavoussi LR, Peters CA (2020) Campbell Walsh Wein urology, 12th edn. Elsevier, Philadelphia

Rabinovich YI, Esayanur M, Daosukho S, Byer KJ, El-Shall HE, Khan SR (2006) Adhesion force between calcium oxalate monohydrate crystal and kidney epithelial cells and possible relevance for kidney stone formation. J Colloid Interface Sci 300(1):131–140. https://doi.org/10.1016/j.jcis.2006.03.070. Epub 2006 Apr 4

Raheem OA, Khandwala YS, Sur RL, Ghani KR, Denstedt JD (2017) Burden of urolithiasis: trends in prevalence, treatments, and costs. Eur Urol Focus 3(1):18–26. https://doi.org/10.1016/j.euf.2017.04.001. Epub 2017 Apr 24

Randall A (1936) An hypothesis for the origin of renal calculus. N Engl J Med 214(6):234–242

Ratkalkar VN, Kleinman JG (2011) Mechanisms of stone formation. Clin Rev Bone Miner Metab 9(3–4):187–197

Rodgers AL, Allie-Hamdulay S, Jackson G, Tiselius HG (2011) Simulating calcium salt precipitation in the nephron using chemical speciation. Urol Res 39(4):245–251. https://doi.org/10.1007/s00240-010-0359-1. Epub 2011 Jan 20

Sheng X, Jung T, Wesson JA, Ward MD (2005) Adhesion at calcium oxalate crystal surfaces and the effect of urinary constituents. Proc Natl Acad Sci U S A 102(2):267–272. https://doi.org/10.1073/pnas.0406835101. Epub 2004 Dec 29

Stoller ML, Meng MV, Abrahams HM, Kane JP (2004) The primary stone event: a new hypothesis involving a vascular etiology. J Urol 171(5):1920–1924. https://doi.org/10.1097/01.ju.0000120291.90839.49

Taylor ER, Stoller ML (2015) Vascular theory of the formation of Randall plaques. Urolithiasis 43 (Suppl 1):41–45. https://doi.org/10.1007/s00240-014-0718-4. Epub 2014 Dec 5

Tiselius HG (1996) Solution chemistry of supersaturation. In: Coe FL, Favus MJ, Pak CYC, Parks JH, Preminger GM (eds) Kidney stones: medical and surgical management. Lippincott-Raven Publishers, Philadelphia, pp 33–64

Tiselius HG (2011) A hypothesis of calcium stone formation: an interpretation of stone research during the past decades. Urol Res 39(4):231–243. https://doi.org/10.1007/s00240-010-0349-3. Epub 2011 Jan 19

Tiselius HG (2013) The role of calcium phosphate in the development of Randall's plaques. Urolithiasis 41(5):369–377. https://doi.org/10.1007/s00240-013-0602-7. Epub 2013 Aug 21

Tsujihata M, Momohara C, Yoshioka I, Tsujimura A, Nonomura N, Okuyama A (2008) Atorvastatin inhibits renal crystal retention in a rat stone forming model. J Urol 180(5): 2212–2217. https://doi.org/10.1016/j.juro.2008.07.024. Epub 2008 Sep 20

Verkoelen CF, Van Der Boom BG, Romijn JC (2000) Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. Kidney Int 58(3): 1045–1054. https://doi.org/10.1046/j.1523-1755.2000.00262.x

Zuo J, Khan A, Glenton PA, Khan SR (2011) Effect of NADPH oxidase inhibition on the expression of kidney injury molecule and calcium oxalate crystal deposition in hydroxy-L-proline-induced hyperoxaluria in the male Sprague-Dawley rats. Nephrol Dial Transplant 26(6): 1785–1796. https://doi.org/10.1093/ndt/gfr035. Epub 2011 Mar 4

Stem Cells: Medical Marvel in Management of Kidney Diseases

16

Shruti Tomar, Veena Puri, Seemha Rai, R. C. Sobti, and Sanjeev Puri

Abstract

Renal response to acute and chronic injury is an intricate process including a vast range of interacting molecules and is indeed a daunting task to tackle. Unilateral ureter obstruction is an excellent model to study kidney injury as it generates fibrosis and extracellular matrix deposition expeditiously leading to end-stage renal disease. Currently, hemodialysis and renal transplantation is the only alternative under such circumstances. However, the fact is that, there is both pessimism and optimism surrounding these treatment modalities in overhauling the damaged tissue. There is, thus, immense clinical need to search for a treatment that can be used without prompting any adverse effects. In this review, we have cast light on the potential attributes of mesenchymal stem cells for the prevention and management of kidney diseases which has attracted a lot of attention recently. Mesenchymal stem cells have proved to be one of the most appealing treatments in regenerative medicine due to their easy accessibility and versatility in action. Thus, stem cells have potential to overcome the inherent limitations of clinical treatment and open new horizons for the treatment of kidney diseases. We summarize recent findings on the administration of mesenchymal stem cells as

S. Tomar · S. Rai (✉)
Centre For Stem Cell Tissue Engineering And Biomedical Excellence, Panjab University, Chandigarh, India
e-mail: seemharai@pu.ac.in

V. Puri
Centre for Systems Biology and Bioinformatics, Punjab University, Chandigarh, India

R. C. Sobti
Department of Biotechnology, Punjab University, Chandigarh, India

S. Puri
Biotechnology Branch, UIET, Punjab University, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_16

a therapeutic agent for renal fibrosis in the context of unilateral ureter obstruction experimental model. Besides, a slight discussion on the role of epithelial-mesenchymal transition during fibrosis is also provided.

Keywords

Unilateral ureter obstruction · Tissue regeneration · Mesenchymal stem cells · Epithelial-mesenchymal transition

16.1 Introduction

Clinical therapy is the mainstay of treatment of severely diseased due to trauma, accidents, or congenital defects, to facilitate tissue repair or regeneration. Tissue or organ transplantations are the first choice for such kinds of diseases which came as an important breakthrough in the medical field. While these treatments have been revolutionary and lifesaving, major problems exist with these techniques like donor deficit, lifelong requirement of immunosuppressants, and other hazardous complications. One of the common instances of such treatments is patients suffering from renal disease which is tremendously affecting individuals' physical as well as mental well-being (Xie et al. 2018). In a way, kidneys are custodian of the human body. Using their tiny nephrons, the kidneys regulate fluid balance in the body to keep our blood healthy and are indispensable for tissue homeostasis. However, the efficiency of the kidneys decreases as the age increases. Though diabetes and hypertension are the main causes that are accountable for kidney diseases, there are also several other factors that ultimately result in loss of renal structure and function (Levin et al. 2017). Compared to other organs in the body, the disease affliction in renal tissue occurs at two different levels the acute renal disease and/(or) chronic renal disease. Acute kidney injury acts as a predisposing factor for the chronic kidney disease ending in end-stage renal disease. The occurrence of reported cases of acute kidney injury (AKI) has increasing at an alarming rate in recent years (Sawhney et al. 2017). Accordingly, the frequency of chronic kidney disease is also progressing that has global implications for health and disease (Heung et al. 2016). As per the study of Global Burden of Disease (GBD) 2017, the frequency of CKD has mounted to 27% from the year 1990 to 2017 (James et al. 2018). Thus, CKD is certain to be one of the most challenging health problems of this century.

The pathogenesis of CKD is based on the deposition of extracellular matrix which leads to fibrosis and finally to end-stage renal disease. Hence, it is a major public health problem and has a considerable impact on billions of individuals worldwide. At present, there is no special treatment for patients suffering from renal interstitial fibrosis other than hemodialysis and renal transplantation. Since renal replacement therapy is a high-priced procedure and most of the sufferers are prevented from being given access to this type of therapy (Liyanage et al. 2015). Therefore, it is high time to look for adequate treatment modalities to delay the renal disease progression.

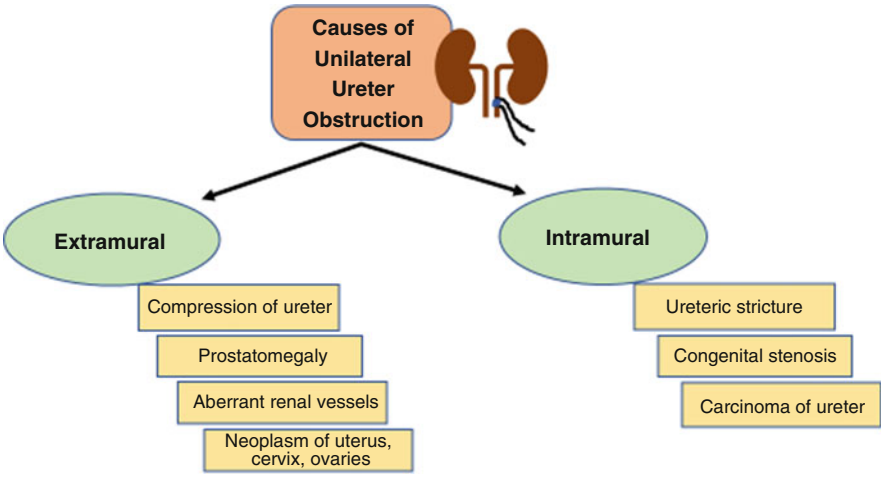


Fig. 16.1 Causes of unilateral ureter obstruction. The figure represents the most common causes of unilateral ureter obstruction

Amongst the numerous causes of kidney failure, unilateral ureter obstruction is one of the potential factors that can be blamed for this disaster. It causes subacute kidney injury peculiarly in infants and children. Ureter obstruction is a serious problem and happens when the flow of urine is prevented due to some blockage in the ureter, the consequence of which is cellular apoptosis and necrosis as an end sequalae to extracellular matrix deposition (Zhang et al. 2018). In order to understand the procedure concerned with the development of kidney fibrogenesis to kidney fibrosis wide variety of models have been explored (Fogo 2001; Rabe and Schaefer 2016). Unilateral Ureter Obstruction (UUO) is scrutinized as one of the extensively studied models for renal injury as it sits at the interface linking AKI and CKD (Wongmekiat et al. 2013). This model has a fair advantage over other models as it generates fibrosis and extracellular matrix development expeditiously. Moreover, by using this model severity and the span of obstruction can be altered according to the requirement (Tan et al. 2007). Additionally, the presence of contralateral kidney in this model can be considered as a control. Human obstructive nephropathies and UUO animal model show many resemblances in terms of manner of causation which puts this model in a favorable position for studying the mechanism of human nephropathy (Klahr and Morrissey 2002; Lopez-Novoa et al. 2010).

The most common causes overall are (Fig. 16.1):

- Extramural: Compression of ureter, prostatomegaly, abnormal growth of renal vessels, neoplasm of adjacent structures like uterus, cervix, ovaries, etc.
- Intramural: Ureteric stricture, congenital stenosis, carcinoma of ureter.

16.2 Obstructive Nephropathy Pathophysiology

Ureter obstruction, although initiated by acute kidney injury can complicate the course and consequently results in end-stage renal disease depending upon the degree and span of obstruction. Ureter obstruction exhibits triphasic design of renal blood flow and pressure changes. Diminishing GFR is the hallmark of acute unilateral obstruction. Immediately after the onset of the first stage of acute unilateral obstruction, which is just after an hour, there is increase in the RBF, accompanied by high renal tubular pressure and collecting system pressure which is imparted to the Bowman's capsule. However, a compensatory response of contralateral non-obstructed kidney intended to maintain GFR. After 2–4 h of obstruction, the pressure remains elevated but renal blood flow and GFR begins to decline due to persistent obstruction. Since the pressure filtration system in the glomerulus is reduced, there is a further decline in RBF and GFR (Farris and Colvin 2012). Leukocyte infiltrate starts appearing in the peritubular interstitium of the injured kidney after 4 h of unilateral obstruction and reaches peak at fourteenth day of obstruction. Leukocyte is accompanied by high renal tubular pressure and collecting system pressure which is imparted to the Bowman's capsule. Leucocyte infiltrate mainly composed of macrophages and T lymphocytes. The leucocyte infiltrate which is recruited at the peritubular interstitium gets accumulated possibly by the production of inflammatory cytokines and growth factors (Chevalier 2006; Li et al. 2018). TNF-α trigger recruitment of leucocytes in the direction of kidney injury mainly at the tubular region of the kidney (Lee et al. 2014).

If the obstruction is not relieved and is maintained then the established kidney lesion may become converted into a progressive destructive lesion which is sorted into three major headings: nephritis, apoptosis of tubular cells, and fibrosis of renal interstitium (Kido et al. 2017; Chaabane et al. 2013). In the development of obstructive nephropathy, there are diverse groups of cells involved like Ang II, TGFβ, TNF-α, connective tissue growth factor (CTGF), various cytokines, renin-angiotensin system (RAS), nuclear factor-κB (NFκB), fibroblasts, and several proteins. Of these AngII and TGF-β1 are of main focus as they play a substantial role in the advancement of kidney diseases (Fig. 16.2).

Acute inflammation is a vital part of body's immune response to injury for the purpose of initiating tissue repair. However, if this response lingers on it may eventually start damaging tissues and organs as is the case of UUO. One of the prime molecules that is responsible for inflammation during urinary obstruction is AngII and is a prominent mediator for stimulation of number of genes that have a role in renal injury (Chevalier and Cachat 2001). After UUO, RAS system gets triggered, and the after-effect of RAS activation is the migration of NF-κB to the nucleus where it induces expression of genes responsible for inflammation (Abbas et al. 2018). A vicious circle exists between NF-κB and RAS; and amidst NF-κB and TNF-α (Hosseinian et al. 2017). Wnt/β-catenin signaling regulates RAS genes (Wang et al. 2018a). RAS gene promotes fibrosis by activating two pathways, TGF-β/Smad 2/3 complex signaling pathway and Wingless/Int/β-catenin signaling pathway. There is a reinforcing loop since fibrosis promotes activation of RAS and it

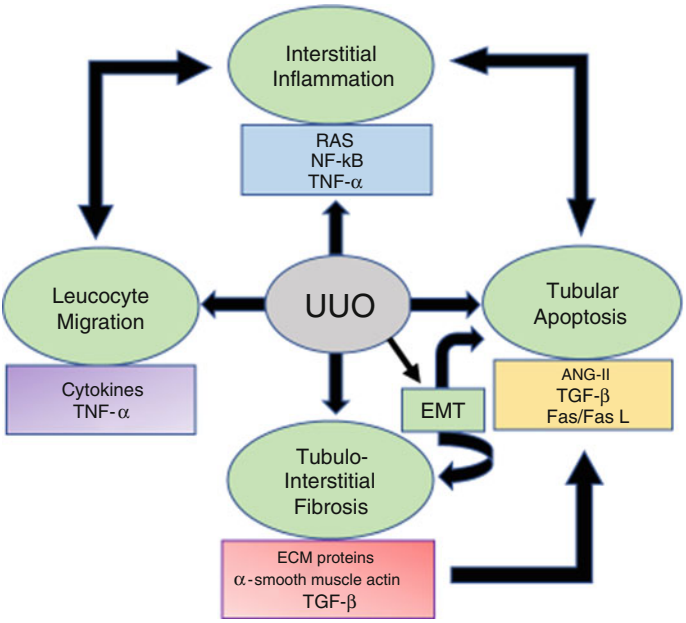


Fig. 16.2 Principal pathogenic mechanisms of unilateral ureter obstruction. The figure depicts the fundamental mechanisms and the primary molecules involved in the pathophysiology of unilateral ureter obstruction

further turns on the activation of above-mentioned signaling pathways, TGF-β/Smad 2/3 complex and Wingless/Int/β-catenin pathways. Chief molecule of RAS system is angiotensin II (Ang II), and most of the physiologic and pathophysiologic functions of angiotensin II are because of the activation of its two receptors, Ang II AT1 (type 1) receptor and Ang II AT2 (type II) receptor (Touyz and Schiffrin 2000; Zhuo et al. 2013).

Apoptosis is a process of programmed cell death that occurs in multicellular organisms when a cell intentionally decides to die. Cellular homeostasis is regulated by a balance among cell proliferation and apoptosis. This is how appropriate cell numbers are maintained in healthy organs. Various forms of cell injury result in an increased rate of apoptosis leading to cell atrophy. UUO is one of the forms of renal injury which results in tubular atrophy due to extensive apoptosis. Mounting evidence indicate that dysregulated apoptosis and EMT have a significant role during UUO-induced kidney injury (Gobe and Axelsen 1987). As a result of EMT, tubular epithelial cells lose their polarity from apical-basal to front-rear and assume a mesenchymal cell phenotype. This architectural rearrangement is the stimulus which prompt cells for the commencement of apoptosis. Tubular cell apoptosis can be perceived after 24 h of obstruction and is at its peak after 12 days of obstruction. Another key factor that is playing a part in apoptosis is mechanical stretching of tubular cells (Chevalier et al. 2010). Onchoprotein Bcl-2, which is well known for its antiapoptotic function is a regulator of apoptosis. Downregulation of

Bcl-2 is an indicator of apoptosis and its expression is diminished in case of dilated tubular cells after UUO (Ucero et al. 2014). It is evident that AngII is a potent pleiotropic molecule that plays a significant role in the commencement of apoptosis by stimulating other factors responsible for the regulation of apoptosis-like TGF-β1, Fas ligand, and caspase activity (Misseri et al. 2004). Elevated level of TGF-β due to sustained obstruction stimulate the production of ROS which is another important mechanism responsible for apoptosis (Hosseinian et al. 2017). Tubular cell apoptosis starts rapidly and is anticipated to amplify with time in case of sustained obstruction (Sun et al. 2013).

Obstructive uropathy consequently leads to kidney fibrosis which gradually terminates in end-stage renal disease if not treated timely. It relentlessly causes amassing of extracellular matrix (ECM) and gradually leads to degeneration of renal function (Soji et al. 2018). This ECM is predominantly produced by α-smooth muscle actin, expressing activated myofibroblasts (Duffield et al. 2013). Apart from α-smooth muscle actin, other proteins that serve as operators of fibrosis progression are type I, type III, and type IV collagen, fibronectin, and glycosaminoglycans (Farris and Alpers 2014). A potentially significant task is played by myofibroblasts in kidney fibrosis; however, their source of production and activation is still debatable. One school of thought presumes them to originate from the local renal fibroblasts itself but according to the study done by Strutz et al., they may arise as a result of EMT causing increased expression of fibroblast markers by the renal tubular epithelial cells under diseased conditions (Strutz et al. 1995). In 2015, two back-to-back studies resolved this argument and proffered additional understanding of the budding role of EMT in the advancement of kidney fibrosis (Grande et al. 2015; Lovisa et al. 2015). Thus, EMT is one of the driving forces behind fibrosis in renal progressive diseases, particularly in UUO model (Hu et al. 2015). Clinical studies have proposed that the acquisition of the α-SMA-positive phenotype by tubular epithelial cells may be secondary to EMT (Zhao et al. 2016).

16.3 Role of Epithelial-Mesenchymal Transition (EMT) in Streaming Kidney Fibrosis

Elizabeth Hay was credited for the first time for introducing the process of epithelial-mesenchymal transition (EMT) using a murine model (Hay 1995). Since it is a reversible process, therefore later, the term transformation was replaced by transition which was concurred in the first meeting of The EMT International Association (TEMTIA) held in Australia in 2003. Fundamentally, the process of EMT involves the transformation of epithelial cells into mesenchymal cells by undergoing manifold biochemical alterations like loss of apical-basal polarity and cell-cell cohesiveness and attain migratory and invasive properties (Kalluri and Weinberg 2009). The mesenchymal cells thus formed have front-rear polarity, spindle-shaped form, and favor cell ECM interaction rather than cell-cell adhesions (Kalluri and Weinberg 2009).

EMT, an evolutionary conserved developmental program is seen during embryogenesis where some of the epithelial cells become pliable and acquire the potential to move to and fro amidst epithelial and mesenchymal states by the process of EMT and MET (Lee et al. 2006). It was further realized that the activation of this program occurs not only during development but also under conditions of wound healing and pathological stress contributing to fibrosis and carcinomas (Kalluri and Weinberg 2009; Piera-Velazquez et al. 2011; Ribatti 2017). As a result, the rising concept of EMT has received a great deal of attraction in the last recent years due to its role not just only in embryology but in pathology as well.

Wound healing is body's innate response to tissue injury and is a dynamic process. Researchers worldwide have identified the role of various cells during tissue repair. Myofibroblasts are one of them which play a significant role during wound healing by degrading the damaged tissue besides synthesizing the provisional ECM (Duffield 2010). After the accomplishment of the wound healing process, myofibroblasts undergo apoptosis and are thus vanished from the injured site. However, sometimes the inflammatory phase is prolonged and the wounds instead of going through the stages of healing remain confined, most probably in the inflammatory phase. Under such instances, myofibroblasts continue to produce fibers, consequently leading to organ fibrosis and ultimately organ destruction (Thannickal et al. 2004).

As mentioned above, that the origin of fibroblasts during fibrosis is a highly debatable issue. Earlier it was assumed that one of the major causes of fibrosis is the persistent generation of interstitial fibroblast which gets transformed into myofibroblasts resulting in scarring of functional tissue (Kriz et al. 2011). However, studies on fibrosis have revealed that a remarkable segment of the myofibroblasts has been contributed by the process of EMT (Iwano et al. 2002). Kidney is one such organ where the role of EMT has been witnessed in conferring fibrosis (Liu 2010). In a model of unilateral ureter obstruction-induced kidney injury, EMT has been observed as a chief process that contributes to fibrosis (Chevalier et al. 2009; Yuan et al. 2015; Lan et al. 2014).

EMT took on a more recognizable form 18 years ago in a research conducted using a mouse model having anti-glomerular membrane disease and was found that kidney epithelial cells abnormally producing fibroblast specific protein (FSP1) (Strutz et al. 1995). It was thus speculated by Strutz et al. that some of epithelial cells gets transformed into fibroblasts during fibrosis. This finding was further certified by Iwano that a considerate number of interstitial fibroblasts are due to epithelial cells of the renal tubule having lac Z as a reporter gene in unilateral ureter obstruction-induced kidney fibrosis in a mouse model (Iwano et al. 2002). These are some of the landmark studies which clearly exemplify the significant part played by EMT in the causation of chronic renal fibrosis in various experimental models.

There are multiple factors responsible for activating EMT in pathological and physiological states (Lim and Thiery 2012). The foremost important factors which play a significant role in triggering EMT includes TWIST, SNAIL, and ZEB (Lamouille et al. 2014; Skrypek et al. 2017; Kishi et al. 2015; Craene and Berx 2013). These factors repress the expression of E-cadherin, thereby leading to loss of

cell-cell adhesions and contributing to initiation of EMT (Peinado et al. 2007). CTGF, IGF, and EGF are also some of the other factors which appear to play important role in induction of EMT and fibrosis (Lim and Thiery 2012; Skrypek et al. 2017). Besides these, TGF-β1 is considered as the principal profibrotic cytokine and myofibroblasts as the dominant cells responsible for generating fibrotic ECM (Ucero et al. 2014; Xia et al. 2018). TGF-β has also proven undoubtedly to be one of the most significant players responsible for inducing EMT (O'Connor and Gomez 2014).

Role of TGF-β is also witnessed in patients who developed fibrosis due to unilateral ureter obstruction and increased expression of TGF-β is seen in renal biopsy of the patients (Sato et al. 2003). In some of the in vitro studies also it was found that the epithelial cells attain the characteristics of mesenchymal cells phenotype when kidney epithelial cell lines were treated with TGF-β (Lamouille et al. 2014; O'Connor and Gomez 2013; Xu et al. 2009). In yet another parallel study, increased expression of α-SMA was observed when rodent mesenchymal cell line MT-9 and a porcine kidney epithelial cell line, LLC-PK1 were treated with TGF-β (Yamate et al. 2005).

Therefore, inhibiting the signaling of TGF-β can be a central target to halt the activation of EMT so as to prevent fibrosis. TGF-β mainly functions through Smad dependent/independent pathway and the signaling molecules predominantly responsible are Smad 2 and Smad 3 (Wang et al. 2005). Upon activation, SMAD proteins form a complex which migrates to the nucleus, ultimately inducing the transcription of their target genes (Saitoh and Miyazawa 2012; Griggs et al. 2017; Hewitson et al. 2017).

It has been revealed by various experiments that BMP-7 plays a significant role in kidney fibrosis by counteracting the action of TGF-β (Long et al. 2013). BMP-7 has an inhibitory effect especially on Smad-3 (Luo et al. 2010), and functions by decreasing the accumulation of ECM and favoring its degradation (Li et al. 2015). Likewise, in UUO model of mouse, deficiency of BMP-6 elevated kidney fibrosis independent of BMP-7 (Dendooven et al. 2011). Thus, not just BMP-7, BMP-6 can also be considered as a potential therapeutic tool (Yan et al. 2009).

Thus, there is enough documentation that EMT plays a key role in renal fibrogenesis by promoting activation and mobilization of multiple fibrogenic cells. The process is mediated through a distinctive signaling pathway which may act as viable therapeutic targets. Therefore, identification of these EMT markers and inhibition of their expression can become central target for antifibrotic strategies. Although a great deal of research has been performed on the application of EMT markers, there is still a great deal that must be achieved in this field so as to use these markers clinically. Moreover, there are no significant differences between the markers of different types of EMT that are used in development as well as in pathology (Popov and Schuppan 2010; Zeisberg and Duffield 2010).

16.4 Intricate Structural Arrangement of Kidney

The mammalian kidney arises from intermediate mesoderm (IM) and passes to three advanced phases during the course of development, pronephros, mesonephros and metanephros. The pronephros and mesonephros gradually degenerate at early stages of the development and the metanephros takes the form of functional and permanent adult kidney (McCampbell and Wingert 2012). Metanephric kidney is produced by two embryonic structures, the ureteric bud and the metanephric mesenchyme (Saxén 1987). The metanephric mesenchyme forms a population stem cell capable of self-renewal called as cap mesenchyme (CM) (Little et al. 2007; Brunskill et al. 2008; Mugford et al. 2009; Yu et al. 2012). Kidney development proceeds when a bundle of CM goes through EMT to form renal vesicles. These renal vesicles formed by CM are the progenitors of nephrons (Saxén 1987; Dressler 2006; Schedl 2007). Nephrons are the structural and functional elements of the kidneys which produce urine and removes wastes from the body. It comprises of three main parts, the glomerulus, tubules, and duct (Reilly et al. 2007).

When the development of adult metanephros kidney is accomplished, it bears an intricate and branched architecture with considerable cellular heterogeneity therefore, their potential of cell renewal is slow and restricted (Reilly et al. 2007). Still, they have the potential to regenerate to a certain extent which further relies on the magnitude of injury. Whenever the kidney gets damaged, the tubules get affected the most as they are highly vulnerable to injury. Following acute kidney injury, the kidney tubules, however, are capable of reestablishing their function. It is proposed that dominating role is played by the kidney stem cells in the repair process which are chiefly present in interstitium or tubules of the kidney. However, this is a remarkably challenging task. As already mentioned above, the kidneys are some of the most important organs having an intricate structure, comprising around 25 different types of cells dispersed in various compartments (Dressler 2006). Therefore, it is surprisingly hard to come up with a precise location of kidney stem cells and to explore their role in tissue repair (Humphreys 2009).

The scenario is totally different when the damage is severe enough, as in the case of chronic injury, which leads to destruction of nephrons and eventually tissue fibrosis. Kidney fibrosis is usually irreversible and consequently triggers toward end stage of the disease. Lifelong dialysis or kidney transplantation is most often required to tackle such problems. Presently, treatment options for renal fibrosis are usually staged as limited (Decleves and Sharma 2014). Therefore, understanding the milieu of the disease is particularly important in order to prevent or revert the progression of the disease. The recent advances in the field of regenerative medicine have motivated many researchers to propose nonrenal stem cells as a versatile treatment for replacement and repair of damaged tissues. In recent years, application of stem cells, specifically mesenchymal stem cells, have proved to be a preferred choice for various disorders (Bianco et al. 2013).

16.5 Stem Cells: Overview

As long as the success of tissue regeneration is concerned, cell source selection plays a very crucial role. It is particularly important to choose an appropriate cell and learn their intricacies to facilitate their effectiveness and success. Since the progress of tissue regeneration relies on the choice of the cell so it becomes mandatory, that the cells should be able to fulfill some of the fundamental requirements before they can be applied clinically. To mention a few, predominantly they should be able to home to the target tissue and should be able to release some signaling molecules for neo-tissue formation. Scientists have targeted almost all the cells in the body for research purposes. Many used autologous chondrocytes, i.e., cells or tissues obtained from the same individual for knee replacement (Mayhew et al. 1998). While for heart valve engineering some utilized nonspecific cell types, including dermal fibroblasts (Shin-Oka et al. 1997). However, while working with such early cell sources, researchers had to confront many challenges due to their severe shortcomings like they got tailored with age and their low yield.

A breakthrough came in the area of tissue regeneration by the probability of using stem cells which paved way for the researchers to design new strategies in the field of regenerative medicine. Stem cells are capable of restoring and repairing damaged tissue. As a consequence, stem cells have come up as promising alternative cell sources for tissue regeneration. Stem cells are considered among the top choices by the researchers not only because of their self-renewal capabilities but also because of their easy accessibility, expansibility, and their potentiality of differentiation (Blanpain and Fuchs 2014).

Together, both embryonic (ESCs) as well as adult (ASCs) stem cells are considered as good sources of stem cells that can be utilized for the applications of tissue regeneration (Bernstein and Srivastava 2012). Both types of cells have their own advantages and disadvantages.

ESCs are isolated from inner cell mass of blastocyst stage of embryo. They are pluripotent cells, i.e., that are able to differentiate into all derivatives of the three primary germ layers—ectoderm, endoderm, and mesoderm—but their use is highly restricted due to ethical controversies associated with them. Isolating the inner cell mass results in destruction of the blastocyst which raises ethical issues. Besides the ethical concerns, there is a technical problem of histocompatibility and their potential to produce teratomas also has to be addressed. All these controversies linked with embryonic stem cells highly prevent their use from participating in the field of tissue engineering.

On the contrary, there are no ethical issues associated with the isolation of adult mesenchymal stromal/stem cells as they reside in the non-embryonic somatic tissues so the destruction of the blastocyst is not involved. The adult stem cells are multipotent cells which have much more lineage restrictions in terms of differentiation potential; but despite that, they succeed in dealing the difficulties that are linked with the embryonic stem cells like ethical issues, negligible chances of tissue rejection, and avoiding teratoma formation. All these advantages together, make the adult stem cells a preferred source for research. Over the past 10 years, the field

of tissue regeneration has been benefitted by the substantial usage of stem cells. Having unique biological properties mesenchymal stem/stromal cells (MSCs) amongst the adult stem cells have been explored widely for research (Kuppe and Kramann 2016).

16.5.1 Mesenchymal Stem Cells Introduction

Both the clinicians and researchers have shown keen interest in the mesenchymal stem cells for their immense potential to enhance tissue regeneration. All stem cells, regardless of their source, share unique properties, such as: they can transform into cells of different types including osteoblasts, adipocytes, chondroblasts, and cells of the visceral mesoderm (Wu et al. 2017a; Shi et al. 2012; Ma et al. 2014). Furthermore, they also keep the capacity of differentiating into the non-mesoderm lineages (Choi et al. 2018; Wan Safwani et al. 2017). Bioactive macromolecules secreted by MSCs are immune-privileged in nature which is also one of the important requirements in the field of tissue repair. Besides, MSCs have the capacity to migrate toward sites of injury and tumor microenvironments. All these properties of stem cells make them potent enough to repair or regenerate any injured tissue and therefore scientists are fascinated to the use of stem cells.

The timeline of the isolation of stem cells marks all the way back in 1967, when Friedenstein and his team, first isolated MSCs from stroma of bone marrow and reported them as plastic-adherent, fibroblast-colony-forming unit cells. These isolated stem cells from stroma of the bone marrow had enormous replicative propensity, great tendency to differentiation into osteoblasts, chondrocytes, adipocytes when cultured in vitro and also had the ability to support hematopoietic microenvironment when individual fibroblast-colony-forming unit cells were in vivo re-transplanted (Friedenstein et al. 1968). Such cells which are presently known as "mesenchymal stem cells" were termed by Arnold Caplan in 1991 (Caplan 1991).

Bone marrow stroma is not the sole source for the isolation of MSCs but there are several other alternative sources from which they can be harvested like: adipose tissue (Wankhade et al. 2016), amniotic fluid (Baulier et al. 2014; Sedrakyan et al. 2012), umbilical cord blood (Bieback et al. 2004), and renal progenitors (Bussolati and Camussi 2015; Pleniceanu et al. 2018) by means of various noninvasive approaches. They can proliferate to enough number for tissue and organ regeneration as they have enormous capacity for self-replication. Owing to these unique properties of MSCs like multi-lineage differentiation potential, immunoregulatory properties, migratory capacity, and ready availability, scientists are taking keen interest in exploring these unique subsets of cells for their potential use in regenerative medicine and tissue engineering.

16.5.2 Benchmarks for Isolation of Stem Cells

Prime issue confronted by the researchers in singling out the MSCs is the existence of varied protocols for harvesting MSCs encompassing multiple laboratories. Therefore, the International Society for Cellular Therapy (ISCT) in 2006 framed a standardized protocol for the selection of MSCs and specified them in accordance with the following parameters:

- Morphologically mesenchymal stem cells should be fibroblast-like cells defined as colony-forming-unit fibroblast (CFU-F) and should be adherent to plastic under standard culture conditions (Dominici et al. 2006).
- MSCs must display the following cell surface markers: CD44, CD73, CD90, and CD105, and diminished levels of MHC-I, and must omit the following set of markers: CD11b, CD14, CD 31, CD34, CD45, and MHCII (Dominici et al. 2006).
- Stem cells should be able to transform in vitro into osteocytes, chondrocytes, and adipocytes (Dominici et al. 2006).

However, based on the above criteria, it is still not possible to isolate MSCs' population which are homogenous in nature and still produce diverse cells. Research is still required to further sort this issue out.

16.5.3 Biological Attributes of Mesenchymal Stem Cells for Kidney Restoration

The mesenchymal stem cells act by multiple mechanisms in restoration of renal injury. On reaching the injured site they differentiate into the renal cells and bring about repair by immunomodulation and also by their paracrine activity (Fig. 16.3).

16.5.3.1 Differentiation

Owing to the multipotent nature of the stem cells they are capable of differentiating into bones, cartilage, fat, tendon, muscle etc. when cultured. In addition to that, MSCs have tremendous plasticity of trans-differentiation once infused into the injured site. Increasing evidence of their differentiation potential is mainly from in vitro reports in contrast to in vivo studies (Weng et al. 2003; Singaravelu and Padanilam 2009; Wong et al. 2014). However, some in vivo studies have been successful in displaying the differentiation capability of MSCs into renal cells. It was demonstrated in a mouse model through laser-scanning microscopy that GFP-tagged bone marrow MSC differentiated into mesangial cells of kidney glomerulus (Imasawa et al. 2001). In yet another research, Li et al. by using ischemic model of mouse further reported the trans-differentiation of infused MSCs toward renal tubular epithelium thereby contributing to tissue recovery (Li et al. 2010). Furthermore, in another model of mouse, it was exhibited that hASC show trans-differentiation into renal tubular epithelial cells at an advanced stage of AKI

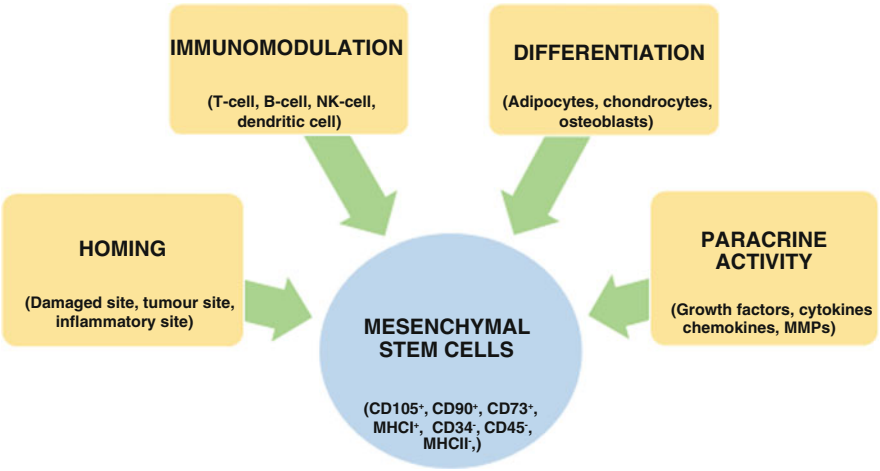


Fig. 16.3 Main processes of mesenchymal stem cell therapy. The figure represents the potential properties of stem cells involved in the treatment of kidney diseases

(Li et al. 2010). Recently, a body of researchers transplanted stem cells from bone marrow of rat into female rats and after 2 days MSCs were found differentiated into embryonic cells (Zou et al. 2016). However, it has been seen in some studies that human bone marrow-derived mesenchymal stem cells, when transplanted into mouse embryo, are capable not only of differentiating into specific renal cells but are also capable of differentiating into a complete nephron (Yokoo et al. 2005). Thus, it has been revealed through several findings that MSC exhibits such traits that can assist in tissue repair/regeneration. Thus, mesenchymal stem cell therapy provides better environment to regenerate damaged cells via differentiating into the renal specific cells and also induce the resident stem cells to regenerate to specific cell types.

16.5.3.2 Homing

Homing is defined as migration of endogenous host cells from their site of storage to a distant organ. Mesenchymal stromal cells, due to their multipotent differential ability, can be directed to migrate to the target areas which can be a tumor site, inflammatory site or even a damaged tissue. Homing of the MSCs may be influenced by the pathological and physiological conditions thus making control of their homing a complex matter.

A team of researchers carried out successful trials and showed the homing of MSCs to the injured kidney after ureter obstruction (Ozbek et al. 2015). Another strong evidence was provided by the researchers where they showed through bioluminescence imaging that the micro-RNA secreted by MSC home the kidney injured by ureter obstruction (Wang et al. 2016). However, in various renal injury models, no proof of homing of cells to injured kidney were detected although therapeutic effect was prominent. In a model of acute kidney injury, there was no

proof of MSCs after 7 days of their infusion (Cheng et al. 2013). Thus, it is a highly debatable issue whether the homing of the infused MSCs to the site of injury is important for their healing action. Most of the studies have shown the clinical efficacy of MSC intravenous delivery but at the same time, following intravenous delivery route of MSC bulk of them are entrapped chiefly in spleen, liver, and lungs (Fischer et al. 2009; Iwai et al. 2014; Tang et al. 2015; Zanetti et al. 2015). As a result, cell count is declined and thus are incapable in reaching the damaged site. Thus, concerted efforts in this area of research are required to ensure maximum homing of cells to the damaged site.

16.5.3.3 Immunomodulation

A breakthrough came with the findings that MSCs have the efficiency to tailor the immune response of an organism which demonstrated that MSCs are immunomodulatory in function (Wang et al. 2018b; Gao et al. 2016). To present that MSCs are immunosuppressive in nature, was first time documented from studies with baboons (Bartholomew et al. 2002) that revealed that activation and proliferation of T cells of our immune system can be repressed by MSCs. Following the first report, consecutive studies were conducted using animal and human models manifesting immunomodulatory property of stem cells. The ability of MSC to dampen the immune response relies on interaction of MSC with the immune cells in conjunction with secretion of soluble factors (Wu et al. 2017b; de Witte et al. 2018). The immune system of a body is the one that has its fair share of controversies against successful outcomes of tissue regeneration applications. It has been shown by various clinical studies that immunomodulation is one of the inherent properties of MSCs and they bear the tendency to:

- Diminish the immune response of T-cells, B-cells, and macrophages (Contreras et al. 2016)
- Modulate the function of T-regulatory cells (Contreras et al. 2016)
- Vanquish the activation of dendritic cells and natural killer cells (Mattar and Bieback 2015).
- Furthermore, they reduce the production of proinflammatory cytokines (Ge et al. 2010; Eggenhofer et al. 2013).

In general, T-regulatory cells and macrophages have been implicated to play a leading role in maintaining the facets of immunomodulatory ability of MSCs (Riquelme et al. 2018; Chang et al. 2012; Goncalves et al. 2017). Thus, due to their immune-privileged status, the role of MSC is just not limited to therapeutic mechanism but are effective across species barriers also (Gieseke et al. 2010). Currently, studies have shown that even the dead and fragmented MSCs retain their immunosuppressive potential (Luk et al. 2016; Koniusz et al. 2016; Nargesi et al. 2017). Apart from interacting with the cells of the immune system they also have the potential to modulate kidney functions like renal blood flow, survival of endothelial cells, and permeability of capillary cells (Kramann and Humphreys 2014). In a recent study, it has been demonstrated that kidney injury has been

ameliorated after infusion of MSCs procured from bone marrow in obstructive nephropathy model by modulating the function of podocytes (Xing et al. 2019).

16.5.3.4 Paracrine Activity

It is no surprise that MSC's differentiation potential and homing at the injured site is strongly correlated to the success of tissue regeneration but it has also been observed that another important mechanism must be responsible for the application of MSC, and paracrine effect has proved to be a potential significant player. Studies have shown that when a tissue gets injured, MSCs secrete a plethora of bioactive molecules like enzymes, growth factors, chemokines, cytokines inclusive of extracellular vesicles, exosomes, and micro-vesicles which are attracted to the damaged tissue to modify its behavior (Andrzejewska et al. 2019). It has also been observed that bioactive factors released by MSCs have proregenerative, antifibrotic, antimicrobial, anti-apoptosis, and antioxidation properties indicating that most of the benefits of MSCs can be attributed to its paracrine effect during tissue injury (Maguire 2013; Haynesworth et al. 1996; Patschan et al. 2006; Tögel et al. 2005; Gnecchi et al. 2006; Kim et al. 2019).

Freshly, it has been shown in various studies that majority of the benefit of MSCs is due the microvesicles secreted by them (Sedrakyan et al. 2017; Bruno et al. 2009; Ranghino et al. 2017). Investigators observed that paracrine activity of extracellular vesicles derived from MSCs relies on the secretion of genes from them that are responsible for angiogenesis (Eirin et al. 2018). In various clinical studies, it has been evidenced that cytokines responsible for inflammation such as TNF-α, IFN-γ, and IL1b are diminished, whereas those suppressing inflammation such as TGF-α and bFGF are highly escalated in kidneys treated with MSCs derived extracellular vesicles (Tögel et al. 2007; Uccelli et al. 2008; Rabb 2005; Hu and Zou 2017).

The potency of extracellular vesicles has been demonstrated in various forms of acute as well as chronic kidney injury. There are some convincing studies in UUO animal model of kidney injury in which the renal injury has been mended by the paracrine effect of EV of MSC (He et al. 2015). Recently, attenuation of kidney fibrosis with prominent decline in the expression of TGFb1, TGFbR1, and collagen IV has been demonstrated in unilateral ureter obstruction-induced kidney injury model by extracellular vesicles secreted by MSCs (Wang et al. 2016). Consequently, evidence suggests that it is the paracrine action of the MSCs that is responsible for conferring renoprotection.

Paracrine effect of MSCs has a profound effect on tissue regeneration and could be a game changer for treating various kidney disorders. Mesenchymal stem cells, therefore, by virtue of their renotropic property and tubular regenerative potential are currently being tested for their potential use in cell and gene therapy for several human debilitating diseases and genetic disorders.

16.6 Concluding Remarks and Future Perspectives

Chronic kidney disease is acknowledged as a considerable medical problem globally and is a crucial issue of public health concern. Kidney possesses a complex architectural structure having an intricate cellular composition which poses challenges to mitigate kidney diseases. Use of stem cells as a curative therapy has become a much-wanted choice now for various types of acute as well as chronic kidney pathologies is gaining ground. MSC form a population of cells that are well distinguished and are easily isolated from a wide variety of human as well animal sources. There are several mechanisms through which MSC exert their therapeutic effect but there are strong evidences which demonstrate that the most promising and effective mechanism of stem cells consists fundamentally in their paracrine and immunomodulatory action. An extensive survey of research supports the restorative efficacy of stem cells in numerous experimental studies of renal disorders and has evidenced outstanding results. Although long-term use of MSCs still remains debatable, therefore researchers are speeding up the pace of this area of research so as to address the flaws associated with stem cell therapy such as mechanism of homing, in vivo tissue differentiation, and tissue-specific delivery of MSCs. Although much has been learned about the therapeutic applications of stem cells, there is still a great deal that has to be achieved before using them clinically and the day is not far when the use of stem cells will speed up exponentially over time, thus paving our way to the most exciting and interesting new frontiers the domain is likely to take in the upcoming future.

References

Abbas NAT, El Salem A, Awad MM (2018) Empagliflozin, SGLT2 inhibitor, attenuates renal fibrosis in rats exposed to unilateral ureteric obstruction: potential role of klotho expression. Naunyn Schmiedebergs Arch Pharmacol 391:1347–1360

Andrzejewska A, Lukomska B, Janowski M (2019) Concise review: mesenchymal stem cells: from roots to boost. Stem Cells 4:1–10

Bartholomew A, Sturgeon C, Siatskas M (2002) Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 30:42–48

Baulier E, Favreau F, Le Corf A et al (2014) Amniotic fluid-derived mesenchymal stem cells prevent fibrosis and preserve renal function in a preclinical porcine model of kidney transplantation. Stem Cells Transl Med 3:809–820

Bernstein HS, Srivastava D (2012) Stem cell therapy for cardiac disease. Pediatr Res 71:491–499

Bianco P, Cao X, Frenette PS et al (2013) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19:35–42

Bieback K, Kern S, Kluter H, Eichler H (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634

Blanpain C, Fuchs E (2014) Stem cell plasticity. Plasticity of epithelial stem cells in tissue regeneration. Science 344:1242281

Bruno S, Grange C, Deregibus MC et al (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. J Am Soc Nephrol 20:1053–1067

Brunskill EW, Aronow BJ, Georgas K, Rumballe B, Valerius MT, Aronow J et al (2008) Atlas of gene expression in the developing kidney at microanatomic resolution. Dev Cell 15:781–791

Bussolati B, Camussi G (2015) Therapeutic use of human renal progenitor cells for kidney regeneration. Nat Rev Nephrol 11(12):695–706

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650

Chaabane W, Praddaude F, Buleon M, Jaafar A, Vallet M, Rischmann P, Galarreta CI, Chevalier RL, Tack I (2013) Renal functional decline and glomerulotubular injury are arrested but not restored by release of unilateral ureteral obstruction (UUO). Am J Physiol Renal Physiol 304: F432–F439

Chang CL, Leu S, Sung HC, Zhen YY, Cho CL, Chen A et al (2012) Impact of apoptotic adipose-derived mesenchymal stem cells on attenuating organ damage and reducing mortality in rat sepsis syndrome induced by cecal puncture and ligation. J Transl Med 10:244

Cheng K, Rai P, Plagov A, Lan X, Kumar D, Salhan D et al (2013) Transplantation of bone marrow-derived MSCs improves cisplatinum-induced renal injury through paracrine mechanisms. Exp Mol Pathol 94:466–473

Chevalier RL (2006) Pathogenesis of renal injury in obstructive uropathy. Curr Opin Pediatr 18: 153–160

Chevalier R, Cachat F (2001) Role of angiotensin II in chronic ureteral obstruction. Contrib Nephrol 135:250–260

Chevalier RL, Forbes MS, Thornhill BA (2009) Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. Kidney Int 75:1145–1152

Chevalier RL, Thornhill BA, Forbes MS, Kiley SC (2010) Mechanisms of renal injury and progression of renal disease in congenital obstructive nephropathy. Pediatr Nephrol 25:687–697

Choi JR, Yong KW, Choi JY (2018) Effects of mechanical loading on human mesenchymal stem cells for cartilage tissue engineering. J Cell Physiol 233:1913–1928

Contreras RA, Figueroa FE, Djouad F, Luz-Crawford P (2016) Mesenchymal stem cells regulate the innate and adaptive immune responses dampening arthritis progression. Stem Cells Int 2016: 3162743

Craene BD, Berx G (2013) Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13:97–110

de Witte SFH, Luk F, Sierra Parraga JM, Gargesha M, Merino A, Korevaar SS et al (2018) Immunomodulation by therapeutic mesenchymal stromal cells (MSC) is triggered through phagocytosis of MSC by monocytic cells. Stem Cells 36:602–615

Decleves A-E, Sharma K (2014) Novel targets of anti-fibrotic and anti-inflammatory treatment in CKD. Nat Rev Nephrol 10:257–267

Dendooven A, van Oostrom O, van der Giezen DM, Leeuwis JW, Snijckers C, Joles JA, Robertson EJ, Verhaar MC, Nguyen TQ, Goldschmeding R (2011) Loss of endogenous bone morphogenetic protein-6 aggravates renal fibrosis. Am J Pathol 178:1069–1079

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

Dressler GR (2006) The cellular basis of kidney development. Annu Rev Cell Dev Biol 22:509–529

Duffield JS (2010) Epithelial to mesenchymal transition in injury of solid organs: fact or artifact? Gastroenterology 139:1081–1083

Duffield JS, Lupher M, Thannickal VJ, Wynn TA (2013) Host responses in tissue repair and fibrosis. Annu Rev Pathol 8:241–276

Eggenhofer E, Popp FC, Mendicino M, Silber P, Van't Hof W, Renner P et al (2013) Heart grafts tolerized through third-party multipotent adult progenitor cells can be retransplanted to secondary hosts with no immunosuppression. Stem Cells Transl Med 2:595–606

Eirin A, Zhu XY, Jonnada S, Lerman A et al (2018) Mesenchymal stem cell-derived extracellular vesicles improve the renal microvasculature in metabolic renovascular disease in swine. Cell Transplant 27:1080–1095

Farris AB, Alpers CE (2014) What is the best way to measure renal fibrosis?: a pathologist's perspective. Kidney Int Suppl 4:9–15

Farris AB, Colvin RB (2012) Renal interstitial fibrosis: mechanisms and evaluation. Curr Opin Nephrol Hypertens 21:289–300
Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI et al (2009) Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev 18:683–692
Fogo AB (2001) Progression and potential regression of glomerulosclerosis. Kidney Int 59(2): 804–819
Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 6(2): 230–247
Gao F, Chiu SM, Motan DA, Zhang Z, Chen L, Ji HL et al (2016) Mesenchymal stem cells and immunomodulation: current status and future prospects. Cell Death Dis 7e:2062
Ge W, Jiang J, Arp J, Liu W, Garcia B, Wang H (2010) Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. Transplantation 90:1312–1320
Gieseke F, Charbonnier LM, Bouffi C et al (2010) Human multipotent mesenchymal stromal cells inhibit use galectin-1 to inhibit immune effector cells. Blood 116:3770–3779
Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20:661–669
Gobe G, Axelsen R (1987) Genesis of renal tubular atrophy in experimental hydronephrosis in the rat. Role of apoptosis. Lab Invest 56(3):273–281
Goncalves FDC, Luk F, Korevaar SS, Bouzid R, Paz AH, Lopez-Iglesias C et al (2017) Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes. Sci Rep 7:12100
Grande MT, Sánchez-Laorden B, López-Blau C, De Frutos CA, Boutet A, Arévalo M et al (2015) Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be targeted to reverse established disease. Nat Med 21:989–997
Griggs LA, Hassan NT, Malik RS, Griffin BP, Martinez BA, Elmore LW, Lemmon CA (2017) Fibronectin fibrils regulate TGF-β1-induced Epithelial-Mesenchymal Transition. Matrix Biol 60–61:157–175
Hay ED (1995) An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 154:8–20
Haynesworth SE, Baber MA, Caplan AI (1996) Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. J Cell Physiol 166:585–592
He J, Wang Y, Lu X, Zhu B et al (2015) Microvesicles derived from bone marrow stem cells protect the kidney in vivo and in vitro by microRNA-dependent repairing. Nephrology 20:591–600
Heung M, Steffick DE, Zivin K et al (2016) Acute kidney injury recovery pattern and subsequent risk of CKD: an analysis of Veterans Health Administration data. Am J Kidney Dis 67:742–752
Hewitson TD, Holt SG, Tan SJ, Wigg B, Samuel CS, Smith ER (2017) Epigenetic modifications to H3K9 in renal tubulointerstitial cells after unilateral ureteric obstruction and TGF-β1 stimulation. Front Pharmacol 8:1–15
Hosseinian S, Rad AK, Bideskan AE, Soukhtanloo M, Sadeghnia H, Shafei MN, Motejadded F, Mohebbati R, Shahraki S, Beheshti F (2017) Thymoquinone ameliorates renal damage in unilateral ureteral obstruction in rats. Pharmacol Rep 69:648–657
Hu H, Zou C (2017) Mesenchymal stem cells in renal ischemia-reperfusion injury biological. Curr Stem Cell Res Ther 12:183–187. https://doi.org/10.2174/1574888X11666161024143640
Hu J, Zhu Q, Li PL, Wang W, Yi F, Li N (2015) Stem cell conditioned culture media attenuated albumin-induced epithelial-mesenchymal transition in renal tubular cells. Cell Physiol Biochem 35:1719–1728
Humphreys BD (2009) Slow-cycling cells in renal papilla: stem cells awaken? J Am Soc Nephrol 20(11):2277–2279

Imasawa T, Utsunomiya Y, Kawamura T, Zhong Y, Nagasawa R, Okabe M (2001) The potential of bone marrow-derived cells to differentiate to glomerular mesangial cells. J Am Soc Nephrol 12: 1401–1409

Iwai S, Sakonju I, Okano S, Teratani T, Kasahara N, Yokote S et al (2014) Impact of ex vivo administration of mesenchymal stem cells on the function of kidney grafts from cardiac death donors in rat. Transplant Proc 46:1578–1584

Iwano M, Plieth D, Danoff TM et al (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 110:341–350

James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N et al (2018) Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the global burden of disease study 2017. Lancet 392(10159):1789–1858

Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420–1428

Kido T, Tsunoda M, Sugaya C, Hano H, Yanagisawa H (2017) Fluoride potentiates tubulointerstitial nephropathy caused by unilateral ureteral obstruction. Toxicology 392:106–118

Kim HK, Lee SG, Lee SW et al (2019) A subset of paracrine factors as efficient biomarkers for predicting vascular regenerative efficacy of mesenchymal stromal/stem cells. Stem Cells 37(1): 77–88

Kishi S, Bayliss PE, Hanai JI (2015) A prospective epigenetic paradigm between cellular senescence and epithelial-mesenchymal transition in organismal development and aging. Transl Res 165:241–249

Klahr S, Morrissey J (2002) Obstructive nephropathy and renal fibrosis. Am J Physiol Renal Physiol 283(5):F861–F875

Koniusz S, Andrzejewska A, Muraca M, Srivastava AK, Janowski M, Lukomska B (2016) Extracellular vesicles in physiology, pathology, and therapy of the immune and central nervous system, with focus on extracellular vesicles derived from mesenchymal stem cells as therapeutic tools. Front Cell Neurosci 10:109

Kramann R, Humphreys BD (2014) Kidney pericytes. Role in regeneration and fibrosis. Semin Nephrol 34:374–383

Kriz W, Kaissling B, Le Hir M (2011) Epithelial-mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy? J Clin Invest 121:468–474

Kuppe C, Kramann R (2016) Role of mesenchymal stem cells in kidney injury and fibrosis. Curr Opin Nephrol Hypertens 25:372–377

Lamouille S, Xu J, Derynck R (2014) Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15:178–196

Lan A, Zhang J, Xiao Z, Peng X, Qi Y, Du J (2014) Akt2 is involved in loss of epithelial cells and renal fibrosis following unilateral ureteral obstruction. PLoS One 9:e105451

Lee JM, Dedhar S, Kalluri R, Thompson EW (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 172:973–981

Lee WC, Jao HY, Hsu JD, Lee YR, Wu MJ, Kao YL, Lee HJ (2014) Apple polyphenols reduce inflammation response of the kidneys in unilateral ureteral obstruction rats. J Funct Foods 11:1–11

Levin A, Tonelli M, Bonventre J, Coresh J, Donner JA, Fogo AB et al (2017) ISN Global Kidney Health Summit participants. Global kidney health 2017 and beyond: a roadmap for closing gaps in care, research, and policy. Lancet 390(10105):1888–1917

Li K, Han Q, Yan X, Liao L, Zhao RC (2010) Not a process of simple vicariousness, the differentiation of human adipose-derived mesenchymal stem cells to renal tubular epithelial cells plays an important role in acute kidney injury repairing. Stem Cells Dev 19(8):1267–1275

Li RX, Yiu WH, Tang SCW (2015) Role of bone morphogenetic protein-7 in renal fibrosis. Front Physiol 6:114

Li YK, Ma DX, Wang ZM, Hu XF, Li SL, Tian HZ, Wang MJ, Shu YW, Yang J (2018) The glucagon-like peptide-1 (GLP-1) analog liraglutide attenuates renal fibrosis. Pharmacol Res 131:102–111

Lim J, Thiery JP (2012) Epithelial-mesenchymal transitions: insights from development. Development 138:3471–3486

Little MH, Brennan J, Georgas K, Davies JA, Davidson DR, Baldock RA et al (2007) A high-resolution anatomical ontology of the developing murine genitourinary tract. Gene Expr Patterns 7:680–699

Liu Y (2010) New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 21:212–222

Liyanage T, Ninomiya T, Jha V (2015) Worldwide access to treatment for end-stage kidney disease. A systematic review. Lancet 385:1975–1982

Long J, Badal SS, Wang Y, Chang BHJ, Rodriguez A, Danesh FR (2013) MicroRNA-22 is a master regulator of bone morphogenetic protein-7/6 homeostasis in the kidney. J Biol Chem 288: 36202–36214

Lopez-Novoa JM, Martinez-Salgado C, Rodriguez-Pena AB, Lopez-Hernandez FJ (2010) Common pathophysiological mechanisms of chronic kidney disease. Therapeutic perspectives. Pharmacol Ther 128(1):61–81

Lovisa S, LeBleu VS, Tampe B, Sugimoto H, Vadnagara K, Carstens JL et al (2015) Epithelial-mesenchymal-transition induces cell cycle arrest and parenchymal damage in renal fibrosis. Nat Med 21:998–1009

Luk F, de Witte SF, Korevaar SS, Roemeling-van Rhijn M, Franquesa M, Strini T et al (2016) Inactivated mesenchymal stem cells maintain immunomodulatory capacity. Stem Cells Dev 25: 1342–1354

Luo DD, Phillips A, Fraser D (2010) Bone morphogenetic protein-7 inhibits proximal tubular epithelial cell Smad3 signaling via increased SnoN expression. Am J Pathol 176:1139–11347

Ma S et al (2014) Immunobiology of mesenchymal stem cells. Cell Death Differ 21(2):216–225

Maguire G (2013) Stem cell therapy without the cells. Commun Integr Biol 6:e26631

Mattar P, Bieback K (2015) Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. Front Immunol 6:560

Mayhew TA, Williams GR, Senica MA, Kuniholm G, Du Moulin GC (1998) Validation of a quality assurance program for autologous cultured chondrocyte implantation. Tissue Eng 4:325–334

McCampbell KK, Wingert RA (2012) Renal stem cells: fact or science fiction? Biochem J 444:153–168

Misseri R, Meldrum DR, Dinarello CA (2004) TNF-alpha mediates obstruction induced renal tubular cell apoptosis and proapoptotic signaling. Am J Physiol 288:F406–F411

Mugford JW, Yu J, Kobayashi A, McMahon AP (2009) High-resolution gene expression analysis of the developing mouse kidney defines novel cellular compartments within the nephron progenitor population. Dev Biol 333:312–323

Nargesi AA, Lerman LO, Eirin A (2017) Mesenchymal stem cell-derived extracellular vesicles for renal repair. Curr Gene Ther 17:29–42

O'Connor JW, Gomez EW (2013) Cell adhesion and shape regulate TGF-1-induced epithelial-myofibroblast transition via MRTF-A signaling. PLoS One 8:e83188

O'Connor JW, Gomez EW (2014) Biomechanics of TGF-induced epithelial-mesenchymal transition: implications for fibrosis and cancer. Clin Transl Med 3:23

Ozbek E, Adas G, Otunctemur A, Duruksu G, Koc B, Polat EC et al (2015) Role of mesenchymal stem cells transfected with vascular endothelial growth factor in maintaining renal structure and function in rats with unilateral ureteral obstruction. Exp Clin Transplant 13:262–272

Patschan D, Plotkin M, Goligorsky MS (2006) Therapeutic use of stem and endothelial progenitor cells in acute renal injury: ca ira. Curr Opin Pharmacol 6:176–183

Peinado H, Olmeda D, Cano A (2007) Snail, ZEB and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7:415–428

Piera-Velazquez S, Li Z, Jimenez SA (2011) Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am J Pathol 179:1074–1080
Pleniceanu O, Omer D, Harari-Steinberg O et al (2018) Renal lineage cells as a source for renal regeneration. Pediatr Res 83:267–274
Popov Y, Schuppan D (2010) Epithelial-to-mesenchymal transition in liver fibrosis: dead or alive? Gastroenterology 139:722–725
Rabb H (2005) Paracrine and differentiation mechanisms underlying stem cell therapy for the damaged kidney. Am J Physiol Renal Physiol 289:29
Rabe M, Schaefer F (2016) Non-transgenic mouse models of kidney disease. Nephron 133:53–61
Ranghino A, Bruno S, Bussolati B et al (2017) The effects of glomerular and tubular renal progenitors and derived extracellular vesicles on recovery from acute kidney injury. Stem Cell Res Ther 8:24
Reilly RF, Bulger RE, Kriz W (2007) Diseases of the kidney and urinary tract. In: Schrier RW (ed) Structural-functional relationships in the kidney, 8th edn. Lippincott Williams & Wilkins, Philadelphia, pp 2–53
Ribatti D (2017) Epithelial-mesenchymal transition in morphogenesis, cancer progression and angiogenesis. Exp Cell Res 353:1–5
Riquelme P, Haarer J, Kammler A, Walter L, Tomiuk S, Ahrens N et al (2018) TIGIT(+) iTregs elicited by human regulatory macrophages control T cell immunity. Nat Commun 9:2858
Saitoh M, Miyazawa K (2012) Transcriptional and post-transcriptional regulation in TGF–mediated epithelial-mesenchymal transition. J Biochem 151:563–571
Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A (2003) Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest 112:1486–1494
Sawhney S, Marks A, Fluck N et al (2017) Intermediate and long-term outcomes of survivors of acute kidney injury episodes: a large population-based cohort study. Am J Kidney Dis 69:18–28
Saxén L (1987) In: Barlow PW, Green PB, Wylie CC (eds) Organogenesis of the kidney. Cambridge University Press, Cambridge
Schedl A (2007) Renal abnormalities and their developmental origin. Nat Rev Genet 8:791–802
Sedrakyan S, Da Sacco S, Milanesi A et al (2012) Injection of amniotic fluid stem cells delays progression of renal fibrosis. J Am Soc Nephrol 23:661–673
Sedrakyan S, Villani V, Da Sacco S et al (2017) Amniotic fluid stem cell-derived vesicles protect from VEGF-induced endothelial damage. Sci Rep 7:16875
Shi Y, Su J, Roberts AI, Shou P, Rabson AB, Ren G (2012) How mesenchymal stem cells interact with tissue immune responses. Trends Immunol 33(3):136–143
Shin-Oka T, Shum-Tim D, Ma P (1997) Tissue-engineered heart valve leaflets—does cell origin affect outcome? Circulation 96:102–107
Singaravelu K, Padanilam BJ (2009) In vitro differentiation of MSC into cells with a renal tubular epithelial-like phenotype. Ren Fail 31:492–502
Skrypek N, Goossens S, De Smedt E, Vandamme N, Berx G (2017) Epithelial-to-mesenchymal transition: epigenetic reprogramming driving cellular plasticity. Trends Genet 33:943–959
Soji K, Doi S, Nakashima A, Sasaki K, Doi T, Masaki T (2018) Deubiquitinase inhibitor PR-619 reduces Smad4 expression and suppresses renal fibrosis in mice with unilateral ureteral obstruction. PLoS One 13:e0202409
Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE et al (1995) Identification and characterization of a fibroblast marker: FSP1. J Cell Biol 130:393–405
Sun D, Bu L, Liu C, Yin Z, Zhou X, Li X, Xiao A (2013) Therapeutic effects of human amniotic fluid-derived stem cells on renal interstitial fibrosis in a murine model of unilateral ureter obstruction. PLoS One 8:e65042
Tan X, Li Y, Liu Y (2007) Therapeutic role and potential mechanisms of active vitamin D in renal interstitial fibrosis. J Steroid Biochem Mol Biol 103(3–5):491–496

Tang Y, Zhang C, Wang J, Lin X, Zhang L, Yang Y et al (2015) MRI/SPECT/fluorescent tri-modal probe for evaluating the homing and therapeutic efficacy of transplanted mesenchymal stem cells in a rat ischemic stroke model. Adv Funct Mater 25:1024–1034

Thannickal VJ, Toews GB, White ES, Lynch JP III, Martinez FJ (2004) Mechanisms of pulmonary fibrosis. Annu Rev Med 55:395–417

Tögel F, Hu Z, Weiss K (2005) Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Ren Physiol 289:31–42

Tögel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C (2007) Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am J Physiol Renal Physiol 292:F1626–F1635

Touyz R, Schiffrin E (2000) Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev 52(4):639–672

Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736

Ucero AC, Benito-Martin A, Izquierdo MC, Sanchez-Niño MD, Sanz AB, Ramos AM, Berzal S, Ruiz-Ortega M, Egido J, Ortiz A (2014) Unilateral ureteral obstruction: beyond obstruction. Int Urol Nephrol 46:765–776

Wan Safwani WKZ, Choi JR, Yong KW, Ting I, Mat Adenan NA, Pingguan-Murphy B (2017) Hypoxia enhances the viability, growth and chondrogenic potential of cryopreserved human adipose-derived stem cells. Cryobiology 75:91–99

Wang W, Koka V, Lan HY (2005) Transforming growth factor-beta and Smad signalling in kidney diseases. Nephrology 10:48–56

Wang B, Yao K, Huuskes BM, Shen HH, Zhuang J, Godson C et al (2016) Mesenchymal stem cells deliver exogenous microrna-let7c via exosomes to attenuate renal fibrosis. Mol Ther 24:1290–1301

Wang M, Chen DQ, Chen L, Cao G, Zhao H, Liu D, Vaziri ND, Guo Y, Zhao YY (2018a) Novel inhibitors of the cellular renin-angiotensin system components, poricoic acids, target Smad3 phosphorylation and Wnt/β-catenin pathway against renal fibrosis. Br J Pharmacol 175:2689–2708

Wang M, Yuan Q, Xie L (2018b) Mesenchymal stem cell-based immunomodulation: properties and clinical application. Stem Cells Int 2018:3057624

Wankhade UD, Shen M, Kolhe R et al (2016) Advances in adipose-derived stem cells isolation, characterization, and application in regenerative tissue engineering. Stem Cells Int 2016:1–9

Weng YS, Lin HY, Hsiang YJ, Hsieh CT, Li WT (2003) The effects of different growth factors on human bone marrow stromal cells differentiating into hepatocyte-like cells. Adv Exp Med Biol 534:119–128

Wong CY, Tan EL, Cheong SK (2014) In vitro differentiation of mesenchymal stem cells into mesangial cells when co-cultured with injured mesangial cells. Cell Biol Int 38:497–501

Wongmekiat O, Leelarungrayub D, Thamprasert K (2013) Alpha-lipoic acid attenuates renal injury in rats with obstructive nephropathy. Biomed Res Int 2013:1–7

Wu Y, Zhou C, Yuan Q (2017a) Role of DNA and RNA N6-adenine methylation in regulating stem cell fate. Curr Stem Cell Res Ther 13:1

Wu Y, Hoogduijn MJ, Baan CC, Korevaar SS, de Kuiper R, Yan L et al (2017b) Adipose Tissue-derived mesenchymal stem cells have a heterogenic cytokine secretion profile. Stem Cells Int 2017:4960831

Xia ZE, Xi JL, Shi L (2018) 3,30-Diindolylmethane ameliorates renal fibrosis through the inhibition of renal fibroblast activation in vivo and in vitro. Ren Fail 40:447–454

Xie Y, Bowe B, Mokad AH et al (2018) Analysis of the Global Burden of Disease study highlights the global, regional, and national trends of chronic kidney disease epidemiology from 1990 to 2016. Kidney Int 94:567–581

Xing L, Song E, Yu CY, Jia XB, Ma J, Sui MS et al (2019) Bone marrow-derived mesenchymal stem cells attenuate tubulointerstitial injury through multiple mechanisms in UUO model. J Cell Biochem 120:9737–9746

Xu J, Lamouille S, Derynck R (2009) TG-induced epithelial to mesenchymal transition. Cell Res 19:156–172

Yamate J, Kuribayashi M, Kuwamura M, Kotani T, Ogihara K (2005) Differential immunoexpressions of cytoskeletons in renal epithelial and interstitial cells in rat and canine fibrotic kidneys, and in kidney-related cell lines under fibrogenic stimuli. Exp Toxicol Pathol 57:135–147

Yan JD, Yang S, Zhang J, Zhu TH (2009) BMP6 reverses TGF-1-induced changes in HK-2 cells: implications for the treatment of renal fibrosis. Acta Pharmacol Sin 30:994–1000

Yokoo T, Ohashi T, Shen JS, Sakurai K, Miyazaki Y, Utsunomiya Y et al (2005) Human mesenchymal stem cells in rodent whole embryo culture are reprogrammed to contribute to kidney tissues. Proc Natl Acad Sci U S A 102:3296–3300

Yu J, Valerius MT, Duah M, Staser K, Hansard JK, Guo JJ et al (2012) Identification of molecular compartments and genetic circuitry in the developing mammalian kidney. Development 139: 1863–1873

Yuan Y, Zhang F, Wu J, Shao C, Gao Y (2015) Urinary candidate biomarker discovery in a rat unilateral ureteral obstruction model. Sci Rep 5:9314

Zanetti A, Grata M, Etling EB, Panday R, Villanueva FS, Toma C (2015) Suspension-expansion of bone marrow results in small mesenchymal stem cells exhibiting increased transpulmonary passage following intravenous administration. Tissue Eng Part C Methods 21:683–692

Zeisberg M, Duffield JS (2010) Resolved: EMT produces fibroblasts in the kidney. J Am Soc Nephrol 21:1247–1253

Zhang J, Xing ZY, Zha T, Tian XJ, Du YN, Chen J, Xing W (2018) Longitudinal assessment of rat renal fibrosis induced by unilateral ureter obstruction using two-dimensional susceptibility weighted imaging. J Magn Reson Imaging 47:1572–1577

Zhao J, Wang L, Cao AL, Jiang MQ, Chen X, Wang Y et al (2016) Decoction ameliorates renal fibrosis via TGF-beta/Smad signaling pathway in vivo and in vitro. Cell Physiol Biochem 38: 1761–1774

Zhuo JL, Ferrao FM, Zheng Y, Li XC (2013) New frontiers in intrarenal renin-angiotensin system: a critical review of classical and new paradigms. Front Endocrinol 4:166

Zou X, Gu D, Xing X, Cheng Z, Gong D, Zhang G et al (2016) Human mesenchymal stromal cell-derived extracellular vesicles alleviate renal ischemic reperfusion injury and enhance angiogenesis in rats. Am J Transl Res 8:4289–4299

"Deciphering the Code of Male Infertility": Genetic Tests, Counseling, and Molecular Basis of Spermatogenic Failure

17

Anuj Sharma, Aditya Prakash Sharma, Japleen Kaur, and Shrawan K. Singh

Abstract

Infertility is defined as the inability to conceive within 1 year of unprotected sexual intercourse. Questions regarding the cause of infertility are few of the most enigmatic ones for the treating physician. Advances in reproductive techniques and insights into the genetic basis of conception have made it possible to reach at a root cause of infertility, in a proportion of cases previously deemed "idiopathic" or "unexplained." There have been extensive refinements in techniques of sperm retrieval along with embryo selection in assisted reproductive techniques in the past decade. Genetic counseling could provide a crucial path to this complex maze of management for the couples seeking treatment for infertility. In this chapter, we discuss the most relevant clinical conditions caused due to genetic abnormalities, the key aspects of genetic counseling, future prospects, and candidate genes, which will pave the way for future research in this intriguing field.

Keywords

Genetics · Male infertility · Y chromosome microdeletions · Karyotyping · CFTR

A. Sharma · A. P. Sharma (✉) · S. K. Singh
Department of Urology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

J. Kaur
Department of Obstetrics and Gynecology, Dr BR Ambedkar State Institute of Medical Sciences (AIMS, Mohali), SAS Nagar, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_17

17.1 Introduction

Infertility is a perplexing condition since time immemorial. About 15% of all couples globally are found to be suffering from infertility and men contribute to 50% of these cases. In about one third of these cases, men exclusively contribute to the problem (Winters and Walsh 2014). This estimate is by far an underestimate of the burden of infertility especially in our part of the world where infertility is still considered a social taboo and couples hesitate from seeking professional assistance.

The carefully coordinated and complex reproductive physiology of both males and females explains the highly heterogeneous etiology of infertility. Primary pathways for infertility in men including sexual differentiation, genitourinary system development, and gametogenesis have been elucidated in various human and animal studies, aiming to establish the genetic basis of infertility.

17.2 Background History

Initial records of documentation of infertility in medical or English literature from the seventeenth century have incriminated solely the woman partner for infertility or barrenness of the couple. Men were simply thought to provide the "seed" and were seldom considered at fault due to the prevalence of patriarchal societal norms (Evans 2016). Evidence for this may be noted in Daniel Sennert's book entitled *Practical Physick: The Fourth Book* as follows: "Hence we may gather, that Barrenness is oftener from a fault in the women than the men: for in men there is nothing required but fruitful Seed spent into a fruitful womb."

Another similar comment made by James McMath in his book *The Expert Mid-wife: A Treatise of the Diseases of Women with Child* reads: "the vile Imputation of Barrenness, rests almost, solely upon them [i.e. women]."

There are scattered documents, which mention about the role of men in reproductive malfunction (Evans 2016). The following excerpt is from the writing of barrenness by John Tanner in *The Hidden Treasures of the Art of Physick* (1659): "Before you try these uncertain conclusions upon the Woman, examine the man, and see if the fault be not in him. It is known thus, if the man be unable to raise his yard, if he want Sperm, if he hath a swelling in his Stones, or if he have the Running of the Reins, he is not fit for Venus School. If the man be of an effeminate Spirit, if he hath no Beard, if he be long casting forth his Seed, and taketh little delight in the act, and the Woman in the act feeleth his Seed cold, be sure the man is unfruitful." The statement to a great extent summarized the faults, which could be there in men contributing to infertility such as erectile dysfunction, orchitis, hypogonadism, etc. However, the literature such as this was sparse and was almost always overshadowed by the populist "fault in women" theories.

We have come a long way from these biblical times, with recognition of semen analysis as the first step in work-up for a couple suffering from infertility. As we know recognition of problem is half the job done. We have slowly evolved, and with

advent of newer methods of molecular genetics and bioinformatics, we are moving towards the era of precision medicine.

The genetic cause of male infertility was recognized in the 1950s when in a patient of Klinefelter's syndrome an extra X chromosome was reported. However, the majority of advancements in this field are just three decades old. Thereafter, cystic fibrosis transmembrane conductance regulator (CFTR) gene and Y chromosomal abnormality were detected and came into the clinical practice (Ferlin et al. 2007). Now with the application of array-based genomic testing and next generation sequencing (NGS) in the genetic testing, we recognize various unknowns pertaining to the disease process.

17.3 Genetic Testing: Need

By an estimate, genetic defects are found in approximately 15% of all males suffering from infertility. With the introduction of assisted reproductive techniques (ART), novel techniques including intracytoplasmic sperm injection (ICSI), and in vitro fertilization (IVF) have found widespread application. Further experience with these techniques has brought up various issues about the genetic basis of disease and concerns over manipulation of natural selection. These methods allow relatively sub-optimal quality sperm to overcome natural selection mechanism. They produce a viable zygote but at a cost of higher probability of inheriting previously unknown mutations, which can have a detrimental impact on the future generation. Although there is no definite evidence of imprinting disorders associated with these procedures, there have been concerns raised regarding negative epigenetic consequences due to hidden reproductive defects with ART (De Rycke et al. 2002; Thompson et al. 2002). Subtle increase in the prevalence of numerical aberrations in the sex chromosomes after ICSI (0.2–0.6%) and autosomal chromosome abnormalities (0.07–0.4%) has been found (Van Steirteghem et al. 2002). However, this apparent higher prevalence is inherently biased because patients who opt for ICSI or other ART might have a higher incidence of genetic abnormalities a priori due to their infertile status. As we mentioned precision medicine earlier, there are now increasing refinements of procedure to ICSI. Mandatory genetic testing of severe oligozoospermic or azoospermic male prior to ICSI and introduction of techniques such as Intra-cytoplasmic morphologically selected sperm injection (IMSI) are becoming a routine. In this technique, the sperm is seen for abnormalities under high-powered magnification for defects and selected. Such procedures are indeed fascinating and helpful in extreme cases and can overcome certain genetic or epigenetic defects. Nevertheless, it is necessary to determine the genetic basis of male factor infertility to develop appropriate screens for abnormal phenotypes and to find out more effective solutions for the infertility queries.

17.4 Genetic Counseling: *Why Is It Necessary*?

Since the discovery of an extra X chromosome in Klinefelter's patients (47, XXY), the literature has been flooded with over 3000 papers on implications of genes and genetics in male infertility (Ferguson-Smith et al. 1957). They have implicated various genetic linkages as well as genes related to male infertility (Foresta et al. 2005). Despite these numbers of putative genes implicated for male infertility, genetic diagnostic testing is confined to a very limited number of genetic disorders due to various reasons. The prime testing includes screening for azoospermia factor (AZF) deletion, karyotyping for chromosomal disorders, cystic fibrosis transmembrane conductance regulator (CFTR) mutation analysis, congenital hypogonadotropic hypogonadism (CHH), and certain less known monomorphic genetic disorders. The advances made in the field have been unable to change the fact that a genetic diagnosis can be achieved in ~4% of all infertile males—a number that has remained unchanged for a couple of decades. The recent rise in detection rates for genes in diseases with a strong genetic basis is attributed to the widespread application of genomic microarray analysis and Next generation sequencing (NGS) (Rocca et al. 2020). With the advent of NGS, there is opening of unchartered territory for looking at genetic aspects of diseases in male infertility as well. As the data is maturing, we are finding newer and newer genes unfolding their effect on the phenotype, their inheritance pattern, and penetrance, thus answering a number of queries for patients kept under the dustbin diagnosis of "Unexplained infertility."

National Society of Genetic Counselors' Task Force Report defines genetic counseling as "the process of helping people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease." It is a comprehensive process, including scrutiny of family charts and medical histories to assess chances of inheritance. It also encompasses educating about preventive strategies, screening tests, and management options to help in making informed choices and adapt to the condition (Rocca et al. 2020).

This process by definition becomes an integral part of infertility care and couples suffering from one or the other genetic disorders must be provided adequate counseling to help them choose the right option, both for purpose of the treatment and for decision making before and during pregnancy after ART. The key points in discussion include whether ART can be used, techniques of sperm harvesting, chances of conception with ART, chances of transmission of said genetic abnormality, need for preimplantation genetic testing, and odds of phenotypic expression in the progeny. In the next section, we discuss the already established disorders for which genetic tests are in use and future aspects of genetic diagnosis in the field of male infertility.

17.5 Genetic Screening in Quantitative Spermatogenic Abnormalities

17.5.1 Karyotype Anomalies

Chromosomal abnormalities are either structural or numerical. The most common chromosomal abnormality associated with male infertility is Klinefelter syndrome (Fig. 17.1). The Klinefelter Syndrome (47, XXY or mosaic 46, XY/47, XXY or higher grade aneuploidy, i.e. 48, XXXY, 49, XXXXY, etc.) with an incidence of 1: 660 in live births and 1:300 in spontaneous abortions is the most frequent sex chromosome aneuploidy (Fig. 17.2). The most typical semen phenotype is azoospermia, however, cryptozoospermia or severe oligozoospermia can also be evident in rare cases like the mosaic forms (Aksglaede and Juul 2013).

The most common structural abnormality is translocations (Robertsonian/reciprocal) or inversions found in men with oligozoospermia (Krausz et al. 2015).

17.5.2 Indication for Testing

The karyotype should be done in patients with quantitative abnormalities in semen analysis. Patients with moderate oligozoospermia (<10 million spermatozoa/mL)

Fig. 17.1 Phenotypic expression of Klinefelter's syndrome in a 25 year male. Note absence of facial hairs and long arm span

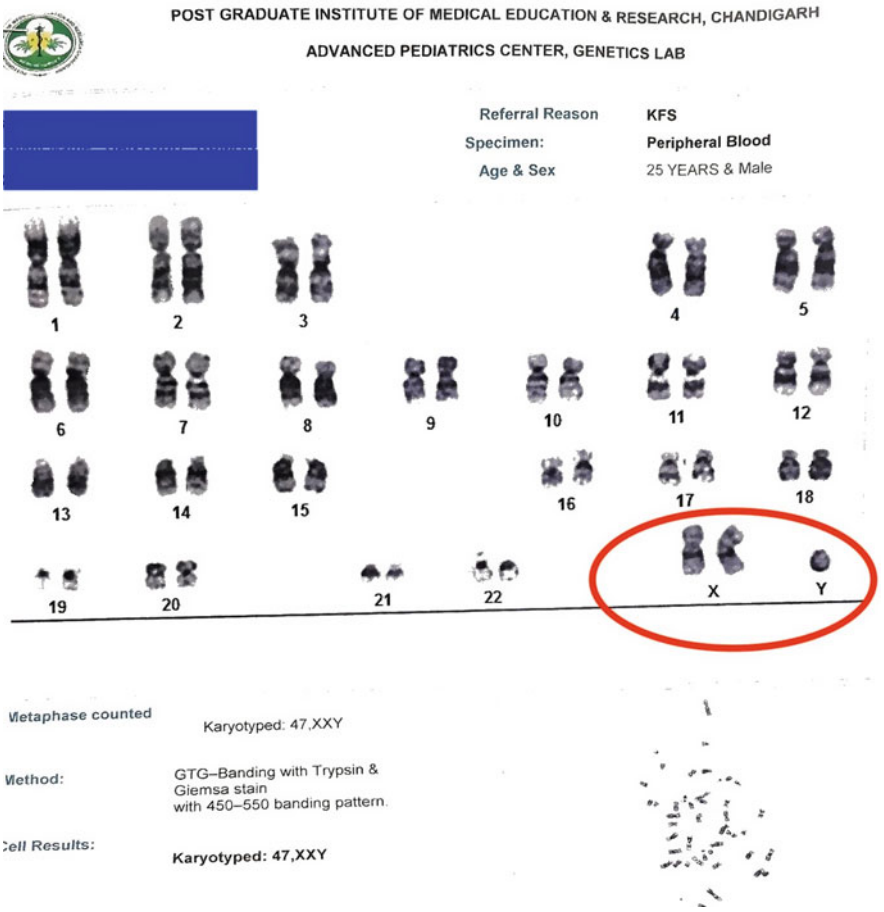


Fig. 17.2 Karyotype of patient of Klinefelter's syndrome

have ten times higher incidence (4%) of autosomal structural anomalies as compared to the general population. This incidence increases to 7–8% in severe oligozoospermia (<5 million spermatozoa/mL) and 15–16% values in NOA (Vincent et al. 2002), indicating that frequency of chromosomal anomalies is directly proportional to severity of the defect in semen analysis. Other indications of karyotyping include recurrent abortions, congenital malformations, multiorgan anomalies, and mental retardation.

17.5.3 Genetic Counseling

Considering age-related progressive germ cell loss, an early diagnosis is a key. MicroTESE (Testicular sperm extraction) has a relatively higher sperm harvesting

rate under 30 years of age (Plotton et al. 2015; Rohayem et al. 2015). The testicular sperm-harvesting rate is 50% and ICSI is the technique favored for the conception of the embryo. Preimplantation Genetic diagnosis (PGD) to prevent the transmission to the offspring is controversial in Klinefelter's. This is because it has not been proven that progeny of patient's with KS have higher chromosomal anomalies (Fullerton et al. 2010).

Further guidance regarding TESE in KS patient, can be guided by the absence of different AZF region as discussed further. There is no higher risk for patients having Y chromosomal structural anomalies. An accurate evaluation of the 45,X cells is needed because mosaicism is seen in these patients representing a poor prognosis for sperm retrieval (Lange et al. 2009).

17.6 Semen Abnormalities due to Y Chromosome Microdeletions

17.6.1 Complete AZF Deletions

Most prominent molecular genetic cause of oligo/azoospermia is Y chromosome microdeletion (Yq, AZF region). There are five different deletion patterns designated as AZFa, AZFb, AZFb+c (with two different breakpoints), and AZFc deletions (Fig. 17.3).

Abnormalities involving the removal of one or more AZF regions have a rare prevalence in the general male population (1:4000) but occur commonly in

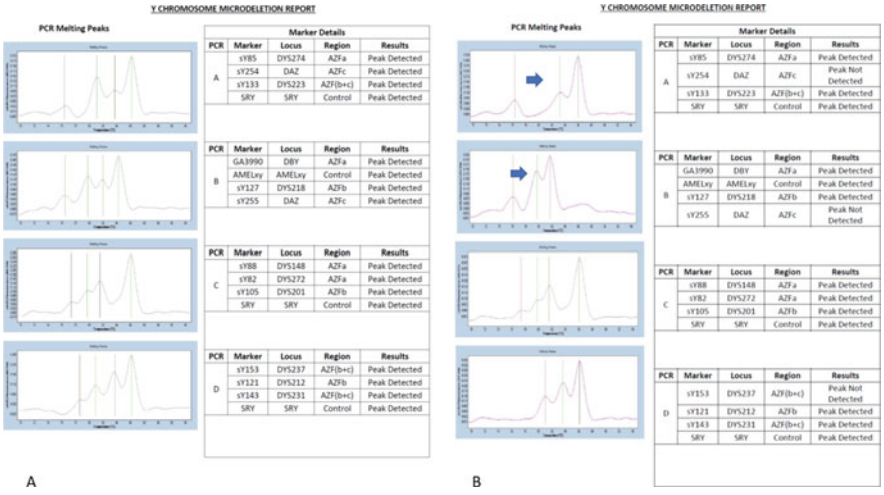


Fig. 17.3 (**a**) Melt curve analysis of the AZF loci markers tested in a patient of severe oligospermia showing no Y chromosome microdeletion. (**b**) Realtime PCR based melt curve analysis in another patient of oligozoospermia indicating deletion of the AZF loci markers sY153 (AZF b + c), sY254 (AZFc), and sY255 (AZFc) in the Y chromosome (Arrow)

idiopathic NOA patients and severely oligozoospermic men (5–10% and 2–5% respectively) (Krausz et al. 2014; Krausz and Casamonti 2017; Lo Giacco et al. 2014). Various studies from India have reported the prevalence estimated at 0–28%. Region-wise distribution provided by Sen et al. showed that South Indian states have higher prevalence of Y chromosomal micro deletions as compared to Northern and Western states (Sen et al. 2013).

AZFa region deletions cause the severest testicular phenotype, which is known as Sertoli cell-only syndrome (SCOS). In this phenotype, the chances of sperm harvesting are almost nil. Azoospermic patients with complete AZFb and AZFb + c deletions may have histological picture corresponding to either SCOS or spermatogenetic arrest (SGA). Residual sperm production may be seen in patients with rare partial AZFa and partial AZFb deletions. AZFc deletions are associated with best prognosis as a variable semen phenotype ranging from oligozoospermia to azoospermia may be noted (Krausz and Casamonti 2017).

17.6.1.1 Indication for Testing

The screening for Y chromosome microdeletions is indicated routinely in the diagnostic work-up of men with severe oligozoospermia (<5 million sperm count) or azoospermia. The methodology for the same is standardized and reported in the European academy of andrology and European molecular genetics quality network (Krausz et al. 2014).

17.6.1.2 Genetic Counseling

Testing for AZF deletions has a prognostic value for testicular sperm retrieval in azoospermic males as mentioned earlier. In complete AZFa and AZFb deletions, probability of finding spermatozoa is almost nil while there is still up to 50% chance of retrieving sperm in men carrying AZFc microdeletion (Lo Giacco et al. 2014). TESE should also be done considering these aspects, hence further defining the need for AZF testing in these males. Moreover, oligozoospermic AZFc deletion carriers should also be explained the option of pre-emptive sperm cryopreservation in young adulthood, because the progressive decrease of sperm production with age has been reported.

17.7 Congenital Absence of Vas Deferens (CAVD)

The CAVD or the associated spectrum of Cystic fibrosis (CF) may be the underlying abnormality in 1–2% of the infertile males and almost one fourth of those with obstructive azoospermia (OA) (Oates and Amos 1993). CAVD is a rare condition associated with oligo/normozoospermia, that can be easily missed if palpation of the vas is not done during routine physical examination. When agenesis of seminal vesicles is associated, it is characterized by typically by low semen volume (<1.0 mL), low pH (<7) along with azoospermia.

17.7.1 CFTR Mutation Screening

The cystic fibrosis transmembrane conductance regulator (CFTR) gene is located on chromosome 7q31.2 (Fig. 17.4). It contains 27 exons and has more than 2000 variants (Riordan et al. 1989). The specific variants and nature of the mutations determine severity of the disease. For example congenital absence of vas deferens occurs when there are two mild mutations or one severe with a mild mutation. On the other hand, a florid cystic fibrosis occurs when two severe mutations occur together in an individual. The most common mutations found in patients with CAVD are 5T, F508 deletion and R117H (Yu et al. 2012). The M470V missense mutation in exon 10 is another phenotype modulating factor (de Meeus et al. 1998).

17.7.1.1 Indication for Testing

Affected patients can have concomitant epididymal malformations, renal and seminal vesicle agenesis, which makes abdomino-pelvic ultrasound necessary in these patients before the genetic testing. Mutational screening is based on the following steps: Initially a targeted variant panel is run, which detects the most common-causing mutational variants with variable rates ranging from 49 to 94% owing to different geographic regions (Lao et al. 2003). If only one or no mutation is found at

Disease Indication: Absence of Left Vas deferens; Infertility.

Result Summary

Test Performed – **CFTR Panel (72 Mutations)**

Results: No variants were detected in sample submitted for analysis

- **WT/WT**
- **Genotype – Undetermined**

72 COMMON *CFTR* MUTATIONS INCLUDED IN THE PANEL

F508del	R553X	1078delT	S549N	D1152H	R75X
I507del	G551D	394delTT	S549R_1645A->C	CFTRdele2.3_3'	S1196X
G542X	1898+1G->A	Y122X	S549R-1647T->G	CFTRdele2.3_5'	W1089X
G85E	2184delA	R347H	V520F	E60X	G1244E
R117H	2789+5G->A	M1101K	A559T	G178R	G1349D
621+1G->T	3120+1G->A	S1255X	1677delTA	G330X	G551S
711+1G->T	R1162X	1898+5G->T	2055del9->A	K710X	R560KT
R334W	3659delC	2183AA->G	2143delT	L206W	S1251N
R347P	3849+10kbC->T	2307insA	3199del6	Q493X	S1255P
A455E	W1282X	Y1092X	3791delC	Q890X	F508C
1717-1G->A	N1303K	3876delA	406-1G->A	R1066C	I507V
R560T	T5/T7/T9	3905insT	935delA	R1158X	I506V

Fig. 17.4 A report depicting a panel of 72 most common CFTR mutations. In this patient of congenital absence of vas deferens no mutations were detected

the first step, next generation sequencing (NGS) of the coding regions and intron-exon boundaries with dosage analysis performed by the normalization of NGS read depth (detection of deletions/duplications) should be considered.

17.7.1.2 Genetic Counseling

Percutaneous epididymal sperm aspiration (PESA)/Testicular sperm aspiration (TESA)/extraction (TESE) combined with ICSI procedure is recommended considering the normal testicular function of congenital bilateral absence of the vas deferens (CBAVD) patients. With a higher carrier frequency for CFTR mutation, screening is also advised in female partners especially in Caucasian ethnicity (1:25). CFTR screening of the spouse of female partner is mandatory in Caucasians, as the carrier frequency of CFTR mutations is high (1:25). When the mutations are found in both the partners, they should be counseled about a very high risk of contracting CF classic form or variant form. Further the need for PGD must be explained to the couple undergoing ART in such a case.

17.7.2 ADGRG2 Mutation Screening

About 20% of the previously unknown mutations in CBAVD patients has been ascertained to a new gene known as G protein-coupled receptor G2 (ADGRG2) gene, which is present on chromosome Xp22.13 and expressed in the efferent ducts (Obermann et al. 2003). In patients of CBAVD, who are negative for CFTR mutation 5 pathogenic ADGRG2 mutations have been found in 11–15% of cases (Obermann et al. 2003; Patat et al. 2016). Thus, in patients who are CFTR negative mutation for ADGRG2 should be checked to look at the X-linked transmission as a cause of CBAVD.

17.8 Congenital Hypogonadotropic Hypogonadism (CHH)

With an incidence of 1:8000 males, CHH is caused due to deficient production or action of gonadotropin releasing hormone (GnRH). The phenotype ranges from the complete form with absent secondary sexual characters (cryptorchidism, micropenis, gynecomastia, inadequate virilization) to partial or late-onset HH. CHH in association with hyposmia/anosmia is termed as Kallmann syndrome (KS). Other associated anomalies like dental agenesis, cleft lip or palate, ear problems, or congenital hearing defects have been reported. If no obvious cause is found in imaging of the brain, it is termed as idiopathic hypogonadotropic hypogonadism. Research into the genetic basis of the disease, especially the idiopathic variety, has identified about 35 candidate genes (Boehm et al. 2015).

17.8.1 Indication for Testing

Other differentials including pituitary tumors, empty sella, etc. need to be ruled out by imaging before clinching to the diagnosis of idiopathic hypogonadotropic hypogonadism. NGS gene panels are used to test for mutated candidate genes, and this provides for the diagnosis in up to 40% of the cases.

17.8.2 Genetic Counseling

The condition is associated with variable inheritance and penetrance. In case of syndromic cases and where an underlying genetic mutation is identified, PGD or prenatal diagnosis can be explained to such couples.

17.9 Qualitative Spermatogenic Abnormalities

Qualitative spermatogenic disturbances are also associated with recurrent mutations. Following rare five phenotypes characterized by autosomal recessive inheritance are currently available for genetic screening: macrozoospermia, globozoospermia, acephalic spermatozoa, multiple morphological abnormalities of the sperm flagella (MMAF), and primary ciliary dyskinesia (PCD).

17.9.1 Macrozoospermia or Sperm Macrocephalia

Sperm with the above-mentioned disorder are large-headed and multi-flagellated (Nistal et al. 1977). This abnormality has been attributed to mutations in AURKC gene encoding for a chromosomal passenger complex (CPC) component in cells undergoing meiosis. The CPC is vital for segregation of chromosomes and for cytokinesis during meiosis (Dieterich et al. 2007). Most frequent (85%) error in the gene is deletion of cytosine in the exon 3 (c.144delC) area.

17.9.1.1 Indication for Testing

Men with macrocephalic spermatozoa can be offered AURKC mutation screening.

17.9.1.2 Genetic Counseling

Men with polypoidal spermatozoa are carriers of homozygous or compound heterozygous mutations. Therefore, ICSI is not advised in these patients, as embryo development is likely to be defective. Male relatives in the family also need to be tested, owing to a high rate of parental consanguinity (Coutton et al. 2015).

17.9.2 Globozoospermia

Sperms with globozoospermia are round-headed and are incapable of fertilizing the oocyte, since acrosomes are absent and no acrosome reaction can occur. This condition is attributed to the four candidate genes (DPY19L2, PBen ICK1, ZPBP, and SPATA16). The most common mutation type is complete deletion (80.4%) (Ben Khelifa et al. 2012).

17.9.2.1 Indication for Testing

DPY19L2 is the most commonly involved gene and should be tested in men with complete globozoospermia. Since gene deletions are not rare, with an incidence of 1: 85 of heterozygous carriers, screening is warranted in the female partners of male carriers before ICSI.

17.9.2.2 Genetic Counseling

ICSI is the only assisted reproductive techniques (ART) option in patients with 100% globozoospermia. However, oocyte activation may not occur in the absence of the acrosome phospholipase C zeta (PLCζ) and is the cause failure for fertilization in these patients. In case if normal spermatozoa cannot be extracted from the ejaculate, the embryologist resort to artificial oocyte activation (AOA) with calcium ionophore.

17.9.3 Acephalic Spermatozoa

Failure of centriole-tail attachment to the spermatid nucleus during the final phase of spermatogenesis, leads to production of acephalic or headless spermatozoa in the ejaculate (Chemes et al. 1987). Underlying biallelic mutations in the SUN5 gene as well as two other new candidate genes (TSGA10 and BRDT) have been identified in various studies (Li et al. 2017; Sha et al. 2018).

17.9.3.1 Indication for Testing

Infertile male patients with acephalic spermatozoa should be offered screening for SUN5 mutations and then counseled for ICSI.

17.9.3.2 Genetic Counseling

ICSI is the last resort for biological paternity in these patients through the selection of tailless sperm heads. It must also be explained to the couple that despite the presence of fertilized eggs, pregnancy rates remain poor.

Various morphological abnormalities with their corresponding genes have been tabulated in Table 17.1.

Table 17.1 Morphological abnormalities with corresponding gene and various recommendation of ART and subsequent counseling

Abnormality	Gene	Recommendation ART	Counseling
Macrozoospermia	AURKC	ICSI not advised	
Globozoospermia	DPY19L2	Artificial oocyte activation (AOA) with calcium ionophore	Pregnancy live birth rate low
Acephalic	SUN5	Select tailless sperm heads	
MMAF	DNAH1	Incomplete asthenozoospermia	
PCD Kartagener's	DNAI1 and DNAH5	ICSI effective	Test female partner PGD if heterozygous
Impaired capacitation	CATSPER1	ICSI	Screen female partner
CAIS/PAIS	AR mutation		Test in case of high ASI (LH × T)

17.9.4 Multiple Morphological Abnormalities of the Sperm Flagella (MMAF)

Multiple morphological abnormalities of the sperm flagella (MMAF) (also known as fibrous sheath dysplasia) is a rare disease characterized by asthenoteratozoospermia. Sperm flagellar abnormalities include absent, angulated, bent, coiled, irregular, or short flagella (Ben Khelifa et al. 2014). Mutations in AKAP3, AKAP4, CFAP43, CFAP44 genes and DNAH1 have been reported. The majority (28–44%) of MMAF patients have biallelic DNAH1 mutations (Amiri-Yekta et al. 2016; Wang et al. 2017). DNAH1 codes for heavy chain of the axonemal heavy arm. Its absence results in a grossly disorganized axoneme which lacks the central pair (9 + 0 structure).

17.9.4.1 Indication for Testing

DNAH1 mutations screening is recommended in patients with sperm flagellar mutations, leading to severe or complete asthenoteratozoospermia.

17.9.4.2 Genetic Counseling

MMAF has been shown to be associated with poor sperm nuclear quality and also increased occurrence of aneuploidies. All mutation carriers with these flagellar abnormalities are not at risk for chromosomal anomalies (Wambergue et al. 2016).

17.9.5 Kartagener's Syndrome or Primary Ciliary Dyskinesia (PCD)

Kartagener syndrome or Primary Ciliary Dyskinesia (PCD), a rare condition, is characterized by severe or total asthenozoospermia associated with malpositioning of internal organs and chronic respiratory infections due to the defects in flagellar and ciliary motility. Ultrastructural defects include microtubular translocations, lack of dynein arms and the missing radial spoke in the centrioles. Twenty-nine genes are

responsible in 70% of the cases of PCD, with DNAI1 and DNAH5 gene mutations accounting for 30% of all case (Knowles et al. 2016; Takeuchi et al. 2018).

17.9.5.1 Indication for Testing

Two most frequently mutated genes are tested initially, followed by multi-gene panel or genomic testing encompassing the 29 possibly responsible genes. As higher prevalence has been documented in consanguineous populations, a targeted analysis for pathogenic variants can represent the first step analysis in certain ethnic groups.

17.9.5.2 Genetic Counseling

ICSI is an effective option for fertility in PCD patients. Owing to the risk of transmission from a heterozygous female carrier, testing of the female before ICSI is recommended and subsequently, PGD can be proposed (Westlander et al. 2003).

17.9.6 Asthenozoospermia

Asthenozoospermia is a perplexing condition for the andrologist, with polygenic inheritance. The error in cation channels of sperm (CATSPER1 and 2) is characterized by the incapacity of sperm in terms of undergoing hyperactivated motility and acrosome reaction (Quill et al. 2001).

17.9.6.1 Indication for Testing

In case of asthenozoospermia, computer assisted sperm analysis (CASA) is used to assess the motility patterns followed by the evaluation of Ca^{2+} influx after progesterone stimulation. CATSPER2-STRC deletion screening needs to be done in men having combination of asthenoteratozoospermia and deafness (i.e. Deafness-Infertility Syndrome).

17.9.6.2 Genetic Counseling

ICSI is the recommended assisted reproductive technique. A high heterozygous carrier frequency of 1.09% for the CATSPER2-STRC gene deletions in general population warrants the need for female partner testing before ICSI (Wang et al. 2017).

17.9.7 Oligo/Azoospermia due to X-Linked Genetic Factors

17.9.7.1 AR (Androgen Receptor) Mutation Screening

Selected cases of quantitative spermatogenic defects are candidates for AR mutation screening. More than 1000 gene mutations have been described in the AR gene, located on Xq11-12 (Gottlieb et al. 2012). The three different phenotypes associated with androgen insensitivity syndrome are: Complete Androgen Insensitivity (CAIS or Morris syndrome), Partial forms (PAI or Reifenstein syndrome), and Mild forms (MAIS). The phenotype varies from beautiful females in patients with CAIS despite

male karyotype, undervirilized males with ambiguous genitalia in PAIS and spermatogenic failure, gynecomastia and hypospadias in MAIS.

The number of CAG repeats in exon 1, together with the gene mutations, appear to modulate the transcriptional activity of the AR protein in vitro. There is a theory that risk for infertility and cryptorchidism is proportional to the length of the CAG repeats, owing to increasing impairment of androgen effect. The range of AR CAG repeat length which predisposes to impaired sperm production and also the relative risk of association of infertility with each length is still debatable. Thus, limiting the clinical use of CAG testing.

Androgen sensitivity index (ASI) is calculated as product of serum testosterone and serum Luteinizing hormone. Males with suspected PAIS or MAIS have a high androgen sensitivity index (ASI) levels. A routine screening to all infertile men is not advised, as the frequency of AR mutations in unselected infertile men is low (0–1.7%) (Rajender et al. 2007). Testing is indicated in patients with high ASI with strong clinical suspicion of AIS.

17.10 Future Directions

One of the primary limitations in the identification of infertility genes remains the rarity of the variants and their phenotypic expression. In the majority of NOA, the rest of the phenotype remains essentially normal. The way to move forward is establishment of consortium and large collaborative trials. International consortia have recently been established in the field of male infertility genetics to promote this, including the GEMINI consortium (https://gemini.conradlab.org) and the IMIGC consortium (http://www.imigc.org). There is a need to collaborate both within these consortia and beyond so as to identify newer mutations, their phenotypic expressions, and decipher the molecular cascade of expression.

With a normal father, the infertile men often wonder and question the inheritance nature of infertility. It is imperative to counsel them that a number of genetic alterations may arise de novo or there may be post zygotic mutations or some mutations may be maternally inherited. Whole exome and genome sequencing of cohorts of patient and parent trios will provide newer insights into these de novo mutations and role of maternal inheritance.

With the new sequencing platforms for reading long length of nucleotides, the researchers are better able to detect repeat expansions, homologous sequences, and structural genomic variation. The limitation of this technology as compared to readily available short sequencing NGS remains its accuracy and cost involved (Hanson et al. 2020; Cannarella et al. 2019). However, the next decade holds the key to affordable human genome sequencing and with use of artificial intelligence and big data analysis, we will enter the era of variant interpretation beyond detection (Chu et al. 2019).

17.11 Concluding Remarks

With the expansion in the horizons of various genetic basis of infertility, the role of preimplantation testing or genetic counseling with couples in our routine infertility clinics has been further strengthened. Gene detection and establishing various disorders of aneuploidy and chromosomal aberrations can explain the previously described "idiopathic" causes of infertility and can certainly answer the questions of couples wishing for conception. Next generation sequencing with newer techniques will help in detection of variants. Large cohort studies and collaborative consortium will help in recording the rare variants and strengthen the field of genetic testing and diagnosis of male infertility, as also help in counseling with respect to transmission to offspring.

References

Aksglaede L, Juul A (2013) Testicular function and fertility in men with Klinefelter syndrome: a review. Eur J Endocrinol 168(4):R67–R76. https://doi.org/10.1530/EJE-12-0934

Amiri-Yekta A, Coutton C, Kherraf ZE, Karaouzène T, Le Tanno P, Sanati MH, Sabbaghian M, Almadani N, Sadighi Gilani MA, Hosseini SH, Bahrami S, Daneshipour A, Bini M, Arnoult C, Colombo R, Gourabi H, Ray PF (2016) Whole-exome sequencing of familial cases of multiple morphological abnormalities of the sperm flagella (MMAF) reveals new DNAH1 mutations. Hum Reprod 31(12):2872–2880. https://doi.org/10.1093/humrep/dew262

Ben Khelifa M, Coutton C, Blum MG, Abada F, Harbuz R, Zouari R, Guichet A, May-Panloup P, Mitchell V, Rollet J, Triki C, Merdassi G, Vialard F, Koscinski I, Viville S, Keskes L, Soulie JP, Rives N, Dorphin B, Lestrade F, Ray PF (2012) Identification of a new recurrent aurora kinase C mutation in both European and African men with macrozoospermia. Hum Reprod 27(11): 3337–3346. https://doi.org/10.1093/humrep/des296

Ben Khelifa M, Coutton C, Zouari R, Karaouzène T, Rendu J, Bidart M, Yassine S, Pierre V, Delaroche J, Hennebicq S, Grunwald D, Escalier D, Pernet-Gallay K, Jouk PS, Thierry-Mieg N, Touré A, Arnoult C, Ray PF (2014) Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella. Am J Hum Genet 94(1):95–104. https://doi.org/10.1016/j.ajhg.2013.11.017

Boehm U, Bouloux PM, Dattani MT, de Roux N, Dodé C, Dunkel L, Dwyer AA, Giacobini P, Hardelin JP, Juul A, Maghnie M, Pitteloud N, Prevot V, Raivio T, Tena-Sempere M, Quinton R, Young J (2015) Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism—pathogenesis, diagnosis and treatment. Nat Rev Endocrinol 11(9):547–564. https://doi.org/10.1038/nrendo.2015.112

Cannarella R, Condorelli RA, Duca Y, La Vignera S, Calogero AE (2019) New insights into the genetics of spermatogenic failure: a review of the literature. Hum Genet 138(2):125–140. https://doi.org/10.1007/s00439-019-01974-1

Chemes HE, Carizza C, Scarinci F, Brugo S, Neuspiller N, Schwarsztein L (1987) Lack of a head in human spermatozoa from sterile patients: a syndrome associated with impaired fertilization. Fertil Steril 47(2):310–316. https://doi.org/10.1016/s0015-0282(16)50011-9

Chu KY, Nassau DE, Arora H, Lokeshwar SD, Madhusoodanan V, Ramasamy R (2019) Artificial intelligence in reproductive urology. Curr Urol Rep 20(9):52. https://doi.org/10.1007/s11934-019-0914-4

Coutton C, Escoffier J, Martinez G, Arnoult C, Ray PF (2015) Teratozoospermia: spotlight on the main genetic actors in the human. Hum Reprod Update 21(4):455–485. https://doi.org/10.1093/humupd/dmv020

de Meeus A, Guittard C, Desgeorges M, Carles S, Demaille J, Claustres M (1998) Linkage disequilibrium between the M470V variant and the IVS8 polyT alleles of the CFTR gene in CBAVD. J Med Genet 35(7):594–596. https://doi.org/10.1136/jmg.35.7.594

de Rycke M, Liebaers I, Van Steirteghem A (2002) Epigenetic risks related to assisted reproductive technologies: risk analysis and epigenetic inheritance. Hum Reprod 17(10):2487–2494. https://doi.org/10.1093/humrep/17.10.2487

Dieterich K, Soto Rifo R, Faure AK, Hennebicq S, Ben Amar B, Zahi M, Perrin J, Martinez D, Sèle B, Jouk PS, Ohlmann T, Rousseaux S, Lunardi J, Ray PF (2007) Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. Nat Genet 39(5):661–665. https://doi.org/10.1038/ng2027

Evans J (2016) 'They are called Imperfect men': male infertility and sexual health in early modern England. Soc Hist Med 29(2):311–332. https://doi.org/10.1093/shm/hku073

Ferguson-Smith MA, Lennox B, Mack WS, Stewart JS (1957) Klinefelter's syndrome; frequency and testicular morphology in relation to nuclear sex. Lancet 273(6987):167–169. https://doi.org/10.1016/s0140-6736(57)90617-7

Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G, Foresta C (2007) Male infertility: role of genetic background. Reprod Biomed Online 14(6):734–745. https://doi.org/10.1016/s1472-6483(10)60677-3

Foresta C, Garolla A, Bartoloni L, Bettella A, Ferlin A (2005) Genetic abnormalities among severely oligospermic men who are candidates for intracytoplasmic sperm injection. J Clin Endocrinol Metab 90(1):152–156. https://doi.org/10.1210/jc.2004-1469

Fullerton G, Hamilton M, Maheshwari A (2010) Should non-mosaic Klinefelter syndrome men be labelled as infertile in 2009. Hum Reprod 25(3):588–597. https://doi.org/10.1093/humrep/dep431

Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M (2012) The androgen receptor gene mutations database: 2012 update. Hum Mutat 33(5):887–894. https://doi.org/10.1002/humu.22046

Hanson BM, Kaser DJ, Franasiak JM (2020) Male infertility and the future of in vitro fertilization. Urol Clin North Am 47(2):257–270. https://doi.org/10.1016/j.ucl.2019.12.012

Knowles MR, Zariwala M, Leigh M (2016) Primary ciliary dyskinesia. Clin Chest Med 37(3):449–461. https://doi.org/10.1016/j.ccm.2016.04.008

Krausz C, Casamonti E (2017) Spermatogenic failure and the Y chromosome. Hum Genet 136(5):637–655. https://doi.org/10.1007/s00439-017-1793-8

Krausz C, Hoefsloot L, Simoni M, Tüttelmann F, European Academy of Andrology, & European Molecular Genetics Quality Network (2014) EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. Andrology 2(1):5–19. https://doi.org/10.1111/j.2047-2927.2013.00173.x

Krausz C, Escamilla AR, Chianese C (2015) Genetics of male infertility: from research to clinic. Reproduction 150(5):R159–R174. https://doi.org/10.1530/REP-15-0261

Lange J, Skaletsky H, van Daalen SK, Embry SL, Korver CM, Brown LG, Oates RD, Silber S, Repping S, Page DC (2009) Isodicentric Y chromosomes and sex disorders as byproducts of homologous recombination that maintains palindromes. Cell 138(5):855–869. https://doi.org/10.1016/j.cell.2009.07.042

Lao O, Andrés AM, Mateu E, Bertranpetit J, Calafell F (2003) Spatial patterns of cystic fibrosis mutation spectra in European populations. Eur J Hum Genet 11(5):385–394. https://doi.org/10.1038/sj.ejhg.5200970

Li L, Sha Y, Wang X, Li P, Wang J, Kee K, Wang B (2017) Whole-exome sequencing identified a homozygous BRDT mutation in a patient with acephalic spermatozoa. Oncotarget 8(12):19914–19922. https://doi.org/10.18632/oncotarget.15251

Lo Giacco D, Chianese C, Sánchez-Curbelo J, Bassas L, Ruiz P, Rajmil O, Sarquella J, Vives A, Ruiz-Castañé E, Oliva R, Ars E, Krausz C (2014) Clinical relevance of Y-linked CNV screening in male infertility: new insights based on the 8-year experience of a diagnostic genetic laboratory. Eur J Hum Genet 22(6):754–761. https://doi.org/10.1038/ejhg.2013.253

Nistal M, Paniagua R, Herruzo A (1977) Multi-tailed spermatozoa in a case with asthenospermia and teratospermia. Virchows Arch B Cell Pathol 26(2):111–118. https://doi.org/10.1007/BF02889540

Oates RD, Amos JA (1993) Congenital bilateral absence of the vas deferens and cystic fibrosis. A genetic commonality. World J Urol 11(2):82–88. https://doi.org/10.1007/BF00182034

Obermann H, Samalecos A, Osterhoff C, Schröder B, Heller R, Kirchhoff C (2003) HE6, a two-subunit heptahelical receptor associated with apical membranes of efferent and epididymal duct epithelia. Mol Reprod Dev 64(1):13–26. https://doi.org/10.1002/mrd.10220

Patat O, Pagin A, Siegfried A, Mitchell V, Chassaing N, Faguer S, Monteil L, Gaston V, Bujan L, Courtade-Saïdi M, Marcelli F, Lalau G, Rigot JM, Mieusset R, Bieth E (2016) Truncating mutations in the adhesion G protein-coupled receptor G2 gene ADGRG2 cause an X-linked congenital bilateral absence of vas deferens. Am J Hum Genet 99(2):437–442. https://doi.org/10.1016/j.ajhg.2016.06.012

Plotton I, Giscard d'Estaing S, Cuzin B, Brosse A, Benchaib M, Lornage J, Ecochard R, Dijoud F, Lejeune H, FERTIPRESERVE Group (2015) Preliminary results of a prospective study of testicular sperm extraction in young versus adult patients with nonmosaic 47,XXY Klinefelter syndrome. J Clin Endocrinol Metab 100(3):961–967. https://doi.org/10.1210/jc.2014-3083

Quill TA, Ren D, Clapham DE, Garbers DL (2001) A voltage-gated ion channel expressed specifically in spermatozoa. Proc Natl Acad Sci 98(22):12527–12531. https://doi.org/10.1073/pnas.221454998

Rajender S, Singh L, Thangaraj K (2007) Phenotypic heterogeneity of mutations in androgen receptor gene. Asian J Androl 9(2):147–179. https://doi.org/10.1111/j.1745-7262.2007.00250.x

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245(4922):1066–1073. https://doi.org/10.1126/science.2475911

Rocca MS, Msaki A, Ghezzi M, Cosci I, Pilichou K, Celeghin R, Foresta C, Ferlin A (2020) Development of a novel next-generation sequencing panel for diagnosis of quantitative spermatogenic impairment. J Assist Reprod Genet 37(4):753–762. https://doi.org/10.1007/s10815-020-01747-0

Rohayem J, Fricke R, Czeloth K, Mallidis C, Wistuba J, Krallmann C, Zitzmann M, Kliesch S (2015) Age and markers of Leydig cell function, but not of Sertoli cell function predict the success of sperm retrieval in adolescents and adults with Klinefelter's syndrome. Andrology 3(5):868–875. https://doi.org/10.1111/andr.12067

Sen S, Pasi AR, Dada R, Shamsi MB, Modi D (2013) Y chromosome microdeletions in infertile men: prevalence, phenotypes and screening markers for the Indian population. J Assist Reprod Genet 30(3):413–422. https://doi.org/10.1007/s10815-013-9933-0

Sha YW, Sha YK, Ji ZY, Mei LB, Ding L, Zhang Q, Qiu PP, Lin SB, Wang X, Li P, Xu X, Li L (2018) TSGA10 is a novel candidate gene associated with acephalic spermatozoa. Clin Genet 93(4):776–783. https://doi.org/10.1111/cge.13140

Takeuchi K, Kitano M, Kiyotoshi H, Ikegami K, Ogawa S, Ikejiri M, Nagao M, Fujisawa T, Nakatani K (2018) A targeted next-generation sequencing panel reveals novel mutations in Japanese patients with primary ciliary dyskinesia. Auris Nasus Larynx 45(3):585–591. https://doi.org/10.1016/j.anl.2017.09.007

Thompson JG, Kind KL, Roberts CT, Robertson SA, Robinson JS (2002) Epigenetic risks related to assisted reproductive technologies: short- and long-term consequences for the health of children conceived through assisted reproduction technology: more reason for caution? Hum Reprod 17(11):2783–2786. https://doi.org/10.1093/humrep/17.11.2783

Van Steirteghem A, Bonduelle M, Devroey P, Liebaers I (2002) Follow-up of children born after ICSI. Hum Reprod Update 8(2):111–116. https://doi.org/10.1093/humupd/8.2.111

Vincent MC, Daudin M, De MP, Massat G, Mieusset R, Pontonnier F, Calvas P, Bujan L, Bourrouillout G (2002) Cytogenetic investigations of infertile men with low sperm counts: a 25-year experience. J Androl 23(1):18–45. https://doi.org/10.1002/j.1939-4640.2002.tb02597.x

Wambergue C, Zouari R, Fourati Ben Mustapha S, Martinez G, Devillard F, Hennebicq S, Satre V, Brouillet S, Halouani L, Marrakchi O, Makni M, Latrous H, Kharouf M, Amblard F, Arnoult C, Ray PF, Coutton C (2016) Patients with multiple morphological abnormalities of the sperm flagella due to DNAH1 mutations have a good prognosis following intracytoplasmic sperm injection. Hum Reprod 31(6):1164–1172. https://doi.org/10.1093/humrep/dew083

Wang X, Jin H, Han F, Cui Y, Chen J, Yang C, Zhu P, Wang W, Jiao G, Wang W, Hao C, Gao Z (2017) Homozygous DNAH1 frameshift mutation causes multiple morphological anomalies of the sperm flagella in Chinese. Clin Genet 91(2):313–321. https://doi.org/10.1111/cge.12857

Westlander G, Barry M, Petrucco O, Norman R (2003) Different fertilization rates between immotile testicular spermatozoa and immotile ejaculated spermatozoa for ICSI in men with Kartagener's syndrome: case reports. Hum Reprod 18(6):1286–1288. https://doi.org/10.1093/humrep/deg240

Winters BR, Walsh TJ (2014) The epidemiology of male infertility. Urol Clin North Am 41(1):195–204. https://doi.org/10.1016/j.ucl.2013.08.006

Yu J, Chen Z, Ni Y, Li Z (2012) CFTR mutations in men with congenital bilateral absence of the vas deferens (CBAVD): a systemic review and meta-analysis. Hum Reprod 27(1):25–35. https://doi.org/10.1093/humrep/der377

Translational Epidemiology in Cancer Research: The Less Travelled Path

18

Gurjeet Kaur, Rahul Soloman Singh, Ashutosh Singh, Harvinder Singh, Shweta Sinha, and Bikash Medhi

Abstract

In cancer research, gene discoveries possess the potential for clinical and public health applications. In addition to gene discoveries, there is rapid development of new technologies such as next-generation sequencing (NGS), which leads to swift augmentation of research in the areas of cancer gene expression, pharmaco-genomics, epigenetics, and proteomics. To confiscate advantage of such discoveries, a systematic translational research approach is needed to reform discoveries from the bench to population health impact. Furthermore, there is an escalating number of large-scale clinical cohort studies with follow-up, in which multiple cohorts have provided distinctive opportunities to convey the effects of various genomic, demographic, molecular, clinical, lifestyle, and psychosocial factors on cancer outcomes.

Translational epidemiology provides opportunities to unite various genomics; pharmaco-genomics, epigenetics, and proteomics-based studies forge new inter-disciplinary collaborative ventures.

Epidemiologists scrutinize different research query at the individual, macro environment, and biological levels. It seems that epidemiology has advantages to act as the bridge of gaps for the translational cancer research. However, the current epidemiology research tends to concentrate on social and environmental factor-based hypotheses, thus restricting its potential to coalesce multiple clinical and biological factors. Recently, there are huge efforts in the epidemiology community trying to fill the gaps to strengthen epidemiological and the clinical studies of cancer outcomes and also to reconstruct epidemiology for twenty-first-

G. Kaur · R. S. Singh · A. Singh · H. Singh · S. Sinha · B. Medhi (✉)
Experimental Pharmacology Laboratory (EPL), Department of Pharmacology, Post Graduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_18

century medicine and effective public health use. Therefore, the sophisticated prototype for translational epidemiology is emerging.

We have specifically contour cancer research areas, wherein translational epidemiology may readily accelerate the appropriate integration of genome discoveries into translation of research for precision medicine. Furthermore, the integration of population studies with these validated genomic discoveries carry great promise for clinical health applications in the development of biomarkers, early detection of cancer, improved risk prediction, more precise diagnosis and prognosis estimation as well as designing targeted therapeutic regime.

Keywords

Cancer · Epigenetic · Epidemiology · Pharmacogenomic · Proteomic

18.1 Brief History of Cancer Research

Cancer is heterogeneous complex multifactorial disease that includes gene–gene, gene–environment, and environment–environment interactions in all the different phases of carcinogenic transformation, progression, and treatment (Sellers 2006). With the rapid development of new technologies such as omics and next-generation sequencing (NGS), several biomarkers are identified and utilized for the early disease diagnosis and prognosis. These biomarkers may also influence the effective treatment response against specific cancer types, the associated side effects, the relapse of disease, prognostic and survival outcomes, and development of drug resistance, which primarily occurs due to late effects of cancer treatments (Freedman et al. 2010a). Furthermore, the advances in biotechnology, bioinformatics, and computational biology provide unprecedentedly rich opportunities for generation of big data and genome-editing technology that will contribute to translation and precision of medicine development in cancer (Fu et al. 2019).

In recent years, genomic and proteomic studies revealed highly complex signaling networks, whose alteration leads to compound effect on disease onset and progression (Karczewski and Snyder 2018; Dagogo-Jack and Shaw 2018). However, for deciphering the exact mechanisms, the sophisticated combination and combination and integration of omics data from genomic, transcriptomic, epigenetic, pharmacogenetics, and proteomic analysis are required from different patient cohorts of multiple cancers that can be used to identify potential cancer biomarkers that would be cell type specific (Sondka et al. 2018; Borrebaeck 2017; Xiao et al. 2014; Wang et al. 2014). Some of the genomic biomarkers such as OncotypeDx and Mammaprint assays have been identified and now used in clinical tests for the evaluation of breast cancer patients, whose gene expression profile influences therapy decisions (Cronin et al. 2007; van ‘t Veer et al. 2002). CancerSEEK is a blood test that can detect eight different cancer types by using genomic and proteomic analysis (Cohen et al. 2018). After comparing omics data, JAK-STAT up regulation and TNF signaling was found as key mechanisms for contributing

triple negative breast cancer (TNBC) onset and progression (Karagoz et al. 2015). These results shed light on the translation of cancer biomarkers identified by NGS platforms and targeted protein detection techniques into clinical applications that can pave new paradigm in cancer diagnosis and prognosis.

18.2 Translational Epidemiology

Epidemiology defines as the study of evaluating causes of health outcome and disease in a population (CDC). Epidemiology is of two types: (1) Descriptive epidemiology—that define the occurrence of disease in a given population, (2) Analytical epidemiology—that define the determinant of the disease (Khoury et al. 2010).

Translational research is defined as the transfer of basic research findings, such as mechanisms, biomarkers of the disease to the clinics for prevention, therapy, and diagnosis of the disease. These findings further applied to clinics for evidence-based recommendations, decision-making, implementing, promulgating evidence-based intervention in practice, and the outcome of the intervention in a population (Khoury et al. 2007, 2010; Sung et al. 2003; Westfall et al. 2007; Shah et al. 2016).

"Translational epidemiology" applies the tools of epidemiology in translational research harnessing the power of proteomics, genomics, and metabolomics (Khoury et al. 2010; Shpilberg et al. 1997).

Translational epidemiology checks and characterizes genetic association discovered by candidate genetic studies, the clinical advantage of candidate application, which provides evidence-based recommendation. It evaluates the applicability of the candidate application on the population outcome in terms of its adverse effect, morbidity, and mortality. Further, it also assesses the challenges in the translational process of the candidate application.

In the current scenario, the advent of new genomic technologies such as human genome sequencing provides a great opportunity for translational epidemiology (Lam et al. 2013). With the introduction of post-genome-wide association studies (GWAS), the field of cancer epidemiology has evolved due to technological and methodological advancement, using bioinformatics, transdisciplinary, and multidisciplinary tools (Nhung et al. 2016). Further, the long term follow up in the large cohort studies and consortium provides an opportunity to produce huge data by evaluating demographic, genomic, clinical, psychological factors and lifestyle on cancer risk factors, intervention selection, and outcome in a population. Thus the important application of translational epidemiology is to translate basic discoveries into population-based health benefits (Singer et al. 2016; Lau et al. 2020).

18.3 Translation Gap Need to Be Filled in Translational Epidemiology: Need to Study Translational Epidemiology

As described earlier translational epidemiology, "The epidemiology, is the study of distribution of determinants of health-related events in a defined population" and application of this study to prevent or control health related events (Khoury et al. 2010).

There are two approaches in translational research: (a) Unidirectional approach—finding in the basic research applied to the clinical set-up by performing different step at different time. (b) Bidirectional approach—population or clinical studies should be applied with modified basic research finding, already explained characterizations. The gaps between bench side to bed side already explained, but epidemiology of the disease and distribution of the determinants should be addressed in the future (Augustin 2003).

For examples of translational medicine success stories, Bevacizumab (Avastin®), a humanized monoclonal antibody targeted to VEGF and Imatinib mesylate which antagonise the fusion of Bcr-abl protein in chronic myelogenous leukaemia explains its importance as well (Huguet et al. 2008; Li 2008; Saijo et al. 2003).

To generalize the scientific knowledge, the basic science knowledge in a population can be helped by -omics—proteomics, genomics, and transcriptomics—to understand the population characteristics regarding the disease. Vast technologies in the biomedical science make a new path for developing preventive, prognostic, diagnostic, and therapeutics (Azad et al. 2006). The exploration of disease physiological determinants, i.e. biomarkers that might help to differentiate expression of specific target from diseased and not diseased states, as well as response to particular therapy. For example, the levels of PSA levels in prostate cancer and the levels of CA-125 in case of ovarian cancer (Hermiston and Kirn 2005; Sørlie et al. 2001).

Most common hurdles in the way of cancer therapeutics are less generalizability to different population and more toxicity in comparison to others. Specificity and predictively sorting based on the omics studies would be required to improve rational use, efficacy of therapy, and more optimization of lead compounds. There are numerous targeted compounds that has been generated by collaboration of basic science researcher, clinicians and industrial synthesizers to decrease the toxicity with compliances of efficacy (Woolf 2008). So, the present inadequacy in formulating the effective translational outcome for the diagnosis and treatment of cancer can be fulfilled by incorporating the multi-disciplinary approach of translational epidemiology.

18.4 Opportunities of Translational Epidemiology

Translational epidemiology research has different applications in scientific discoveries to invent new advancement towards regulation, prior detection and cure of disease in public health practice that leads to health effective policies and improved in health outcomes (Garraway 2013). The purpose of this book chapter is

to provide different opportunities of translational epidemiology in the area of cancer that offer to advance cancer precision and genomic medicines that are presently called as precision oncology (Nhung et al. 2016).

There are escalating number of cohort studies and consortium of cohort with long-term follow up. Various cohort studies give opportunities to focus on the effects of various genomic, demographic, molecular, clinical, and psychosocial factors in cancer outcomes. For instance PLCO cancer screening trial, which is for the prostate, lung, colorectal, and ovarian is huge scale, population-based randomized trial with long-term follow ups. These types of huge scale cohort studies provide intense opportunities for the research based on population in disease etiology and in early detection of markers (Fu et al. 2019).

The epidemiological data that can be linked to the electronic medical and health records with doing some efforts to further assess the patient's outcomes. These efforts help with the systematic study of the research rationale, over the entire life span of patients having cancer. The study could enfold topics from susceptibility of cancer, interaction of gene and environment in initiation of cancer, promotion, and cancer treatment regime and lastly the patient's survival, which is henceforth the most important aim of the study. Hence, a single study can be meaningful to evaluate the risk of cancer, selection of treatment, and speculate the treatment response and survival outcomes. The finding can be remarkably translational and will influence the prevention of cancer and treatment directly (Hall et al. 2008). Given progresses in bioinformatics, biotechnology and system or computational biology, there are remarkably abundant opportunities in large data and technology of genome editing to contribute to translation and precision medicine.

18.5 Main Components of Translational Epidemiology

The success of translational epidemiology lies in thoroughly understanding the main components of translational epidemiology that includes collaboration, advent of technology, multilevel analysis, and adequate knowledge. In view of collaboration, the high degree of complexity of disease demands the integration of a broad range of disciplinary knowledge. Cancer is a multistage and multifactorial disease. Therefore, a collective effort is required for large cohort studies, data collection, data harmonization, and population heterogeneity, in order to unveil the underlying mechanism, environmental factors, genetic factors, prevention, and therapies (Fu et al. 2019; Rosenfield 1992). The disciplines such as epidemiology, statistics, healthcare, genomics, environmental health, social science, and many more need to collaborate to refine huge data generated and to provide conclusive results. Further, for long term follow-up of cancer patients, the epidemiologic data and electronic healthcare records need to be linked (Kessel and Norman Anderson American Psychological Association 2008).

Cross-disciplinary collaboration can be established in three ways—Multidisciplinary, interdisciplinary, and transdisciplinary. Multidisciplinary collaboration is the most common and least integrative approach in which each participant works

independently for the common problem, brought together, and compiles results at the end. The overall output of multidisciplinary collaboration might be conclusive but provides a short-lived solution to a problem.

In an interdisciplinary collaboration, the larger teams use techniques and skills to address the common problem and report their work in a discipline-by-discipline sequence. These types of collaboration though provide new insight but sometimes it does not take into consideration the relevant factors due to discipline boundaries (Hall et al. 2012).The cross-disciplinary collaboration can be established in three ways, firstly transdisciplinary collaboration that is also the most integrative involves the transcends of disciplines by the participants to develop a shared visionary framework (Hall et al. 2012; Burgio et al. 2013). For instance, the National Cancer Institute (NCI) has initiated the transdisciplinary tobacco research use centers (TTURC) that enhanced scientific productivity (Verma et al. 2013). Epidemiology and Genomics Research Program (EGRP) at NCI established a network of 49 cancer epidemiology consortia (CEC) that is one of the largest cancer research collaboration, supported by 201 grants. It manifested the unmet capability of this type of consortia that integrated genomic and omics approaches and produced about 3876 publications within a span of 16 years (1995–2011) (Khoury et al. 2012; Ioannidis et al. 2013).

Ultimately, in translational epidemiology, the collaboration between the funding stakeholders and the scientific community is the arbiter in the long term cohort study. The incorporation of new genomics technology and other high-throughput platforms in a cohort study can be decisive. The rapid availability of advanced technology and multi-level analysis of bioinformatics data, that are available in an online data base, i.e. The Cancer Genome Atlas—National Cancer Institute (TCGA-NIH), has greatly transformed the basic research in area of multiple cancer types. These tools have provided a single platform for analyzing, submitting, and retrieving molecular expression and genomics data that have the potential to revolutionize the translational epidemiology.

Another important aspect of translational epidemiology is, the knowledge of using different platform and technology. The knowledge integration is the process to unite the background and information from various sources to speed up the translation of scientific findings into health benefits for the population as well as to the individuals (Lilienfeld 2000). There are three components involved in knowledge integration (Cameron and Jones 1983).

Knowledge Management: It is used for the selecting, curating, storing, and tracking related information.

Knowledge Synthesis: It is used for how to apply technical methods that includes meta analysis, for the detailed systematic review data that are published or unpublished by using a priori rules of evidence and also for the decision and modeling analysis.

Knowledge Translation: This type of knowledge utilized by using synthesized information to stakeholder's discussion, development of guideline, practice, and influence policy and research.

Knowledge integration has a central role in cancer translational epidemiology. At any time point within the continuity, it delivers scientific guidance and application to assess the accuracy and robustness of existing scientific findings, evaluate their involvement for cancer care and prevention and also help in find out the scientific gaps which permit further research (Slamon et al. 1987).

18.6 Reconstructing Epidemiology for Twenty-First-Century Needs

Translation epidemiology need to be reconstructed with collaborative approach to the public health programs that should be directed to characterize the population genomics (pharmacogenomics, transcriptomics, and proteomics) instead of socio-economic studies. Even epidemiologist plays vital role to compliance the preventive, prognosis and treatment policies, but omics data should be consistent with interpretability of targets and has to make sense in generalization or application. Translation epidemiology is the branch of science that completes the translational purpose of a basic research to application.

After recommendation and health policies should be surveillance for different factors social-economic via feedback mechanism to evaluate the real world health outcomes. Concerted effort should be made across disciplines like basic science researcher, clinicians, information technicians, statisticians, and public health (epidemiologist), with well characterized cohort studies with large sample size. Genomic studies integrated with technology like digital and informatics with communication science needed to generate more data with inference to epidemics.

18.7 Proposed Outcome of Translational Epidemiology

In 1984, John Snow illustrated the transcendent role of epidemiology in translational research, when he presented the strong epidemiological evidence of transmission of cholera by water, that ultimately influences the policymakers to remove Broad street pumps and brought about an eventful change in English public health legislation (Slamon et al. 1989; Wright et al. 1989).

HER2/neu (human epidermal growth factor 2) gene has a significant prognostic value in breast cancer cell growth depicted in observational epidemiological studies (Slamon et al. 2001; Nelson et al. 2005; Abernethy et al. 2010). The findings lead to the development of trastuzumab (monoclonal antibody) against tumor cells expressing HER2/neu (Shrager and Tenenbaum 2014). This was the first molecularly targeted cancer therapy.

On the basis of evidence-based studies, a recommendation was issued by the US preventive service task force in 2005 for the screening of BRCA gene in women having a family history of BRCA1 or BRCA2 gene mutation (Kris et al. 2014).

The large prospective cohort studies can be used to validate the biomarkers of the disease using phenotyping studies, which are not possible in smaller studies.

Precision oncology systematically utilizes the subject experience in the real-time evidence-based practice that is pivotal for translational research. Integration of epidemiological principles along with observational studies is helpful for precision oncology (Garraway 2013; Sleijfer et al. 2013). The observational studies under the lung cancer mutation consortium (LCMC) conducted on 1007 patients identified 10 important mutations in lung adenocarcinoma using multiplex genotyping. This data is decisive for preparing diagnostic and treatment strategies (Nechuta et al. 2011).

Sometimes, small observational studies conducted in a molecularly homogenous population that evaluate the effect of therapies on specific gene alterations provide sufficient clinically validated and relevant data for carrying out clinical trials (Beasley et al. 2012; Kwan et al. 2012).

Pooling studies are also useful because they enhance efficiency by increasing statistical power. For instance, a pooling project in breast cancer that was an integration of four prospective studies, included 18,000 breast cancer survivors with a variety of tumor subtypes (Pierce et al. 2007), assessed the outcome of physical activity and body mass index on the rate of cancer survival (Madlensky et al. 2011; Caan et al. 2011). These types of studies include a range of exposure in a diverse population, higher efficiency, and increase population size.

Data from randomized controlled trials are important, as it provides answer beyond research questions. For example, a randomized trial on 3000 survivors of early-stage breast cancer assessed the effect of a diet containing fruits, vegetables, fiber-intake, and low-fat diet (de González and Morton 2012). The study did not find any beneficial effect of the diet on breast cancer recurrence or survival (Oeffinger et al. 2006; Hudson et al. 2003) but by using the archived blood samples, the study revealed the association of tamoxifen metabolites and CYP2D6 polymorphism with breast cancer recurrence (Oeffinger et al. 2006).

Cancer epidemiology cohort studies are useful to assess cancer development in healthy individuals, longitudinal studies of cancer survivors, and risk evaluation in survivors (Robison et al. 2009). The Childhood Cancer Survivor Study (CCSS) concluded that almost 73% of survivors of cancer exhibited one chronic condition after 30 years post-treatment (Best et al. 2011), approximately 44% of childhood and young cancer survivors have presented diminished health status at long term follow up (Petricoin III et al. 2002a), and 20% of the cumulative incidence of following neoplasms during 30 years follow up. The study generated about 200 publications in 10 years that presented data on the risk factors, comorbidities, second malignant neoplasms, reproductive health, psychological health, and lifestyle (Petricoin III et al. 2002b). With the advent of genomic technologies, the paradigm has shifted from genotype-phenotype mechanism to completely evaluate the genome for an array of variants and establish an association with a complex phenotype. The epidemiologist employs phenotypic factors with blood-based markers and uses consortia to carry out GWAS. This strategy used to identify genetic variants associated with the disease. The CCSS study integrated GWAS in pediatric Hodgkin lymphoma survivors and identified genomic variants associated with increased risk of second malignancies on exposure to radiation therapy (Freedman et al. 2010b).

Moreover, innovation in mass spectroscopy and sequencing technologies provides an insight in terms of metabolomics, transcriptome, and proteome and provides an opportunity for translational epidemiology. The Mass spectra are produced by the surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) integrated with high through output artificial intelligence-based algorithm that identified protein patterns that can further detect ovarian and prostate cancer (Freedman et al. 2016; Rastogi et al. 2004). Though the technology is in the initial stage but illustrated the important capabilities of chip-based technology in cancer diagnosis.

Finally, there is growing interest in the role of blood-based transcriptome in human disease (Smith 2010). Additionally, the broad expression and relative stability of small circulating RNAs, including but not exclusive to microRNAs, suggests that these regulatory transcripts may have far-reaching importance in disease progression (Thomas and Conti 2004).

18.8 Challenges and How to Overcome the Challenges

To support and sustain the translational cancer epidemiology research, certain factors like infrastructure development, strengthen the capabilities of epidemiological research, and encourage the unbiased and the fruitful collaborations must be among different area should be focused. Considering all of these factors can be a good step towards the region where capacity for cancer research is in growing stage. Here we are going to discuss about some challenges that come across in cancer translational epidemiology and how that challenge can be ruled out.

- The basic challenges which are faced in cancer epidemiology is physical infrastructure that involve enhancing the epidemiological data quality, collection of biological specimen, storage, and processing.
- Another challenge that comes to epidemiologist in different countries is that, high illiteracy rates and absence of official residence among specific parts of the population, follow up might be not easy specifically when mailed questionnaires are presented. This issue can be resolved by having researchers to collect both outcome and exposure data via visit to households (Ogino et al. 2012).
- One of the main challenge that intercept in epidemiological discoveries from translating to disease associated intervention is that a large amount of the discoveries from observational studies that illustrate associations instead of causations. To accelerate the transition to application, novel instruments for demonstrating the causation has experienced the bottleneck effect. To overcome this challenge, there is Mendelian Randomization Analysis (MRA), which is based on Mendel's law of independent assortment, is developed (Spitz et al. 2012; Thun et al. 2012). It merges the classical and genetic epidemiological data to reduce or even remove the potential biases in the associations, therefore inferring causality.

- Very low focus has been paid to how scientists should be trained for transdisciplinary research, as we know that translational cancer research is both interdisciplinary as well as transdisciplinary in nature. However multidisciplinary training is a necessity for the next generation researchers who want to be able to conduct translational cancer research, Next generation epidemiologists may have to gain complete knowledge of epidemiology concerned with cancer, genetic or molecular biology, pathology, statistics, and oncology (Omenn et al. 2012; Frueh 2009).
- Epidemiologist faces challenges in addressing the scientific questions, which require large sample sizes for statistical precision. So epidemiologist needs consortia of well-organized cohort studies that will help the scientist to solve the scientific questions (Little et al. 2005).

18.9 Proposing a Conceptual Hypothetical Model of Integrating Different Aspects of Translational Epidemiology for Diagnostic and Therapeutics

The main purpose of translational epidemiology is to identify the different regimes and management models for translating scientific discoveries into population health impact. The main concept of translational epidemiology refers to the translation of basic research new discoveries to useful clinical applications as "bench to bedside" research, as the "effective translation of the mechanisms, novel knowledge, and innovative techniques produced by advances in basic science research into new approaches for diagnosis, prevention and treatment of disease." The identification of different risk factors for cancer has results in the heightened interest in testing the non-pharmacological interventions or treatments that are based on the methods related to lifestyle modification with the ultimate focus to strengthen reserve.

The prolonged latency duration of cancer development and observational study designs played an dominant role in the translational research that include in the development, validation and evaluation of potent biomarkers, clinical health interventions for the effective screening of cancer, early detection as well as prevention. The advancement of high throughput "-omic" technologies have provided huge opportunities for different observational studies to efficiently plan and develop as well as evaluate gene-treatment ways that are applied to treatment of cancer and precision oncology. The discovery of biomarkers were able to predict the successful as well as weak treatment responses and the development of molecular targeted therapies which is often rendered as a following linear, unidirectional process leading up with evaluation in a randomized controlled trial (RCT) (Omenn et al. 2012).

The major principles of epidemiology for the development of an accurate oncology application that have require precise interpretation of the population of interest, defining both the genomic study and treatment comparisons after being made, and apprehending the possible clinical outcomes that are used to check such comparisons. Attention to these principles, especially the comparisons being made, will help in distinguishing multiple or single prognostic factors (course of

disease) from predictive factors (predicting treatment response and outcomes), Hence will provide the strong foundation to facilitate the successful translational research (Frueh 2009).

For the development of genomic markers that can be prognostic or predictive, strengthen the observational research studies that include the access to different patient profiles and multiple cancer types that otherwise were not certainly eligible for RCTs delineated to address the similar questions. These observational research studies have also allowed the in-depth examination of a large number of sample sets to carefully examine the interaction between the varied and discrete genomic markers as well as treatment variable to predict the effective successful outcomes. However, the observational epidemiology has its own limitations in establishing the causal effects and is highly vulnerable to non-causal explanations observed in gene-based treatment interactions. The variation in the allocation of the treatment, effective dose, and frequency as well as uneven dispensation of risk factors that are associated with outcomes of interest between treated and untreated groups that might allow for the high number of potential disconcert and selection bias (Little et al. 2005). An innate logical difficulty in such trials targeting the ongoing reserve is the necessary time lag between the intervention for ameliorating reserve and the eventual effect in minimizing the risk of recurrence and the development of cancer, which need to be fully conveyed.

In order to fully comprehend the potential of translational epidemiology in basic research practice, it is essential to integrate clinical, demographic, and biochemical population data; basic research information with data-driven evaluations in a well-defined clinical setting in diversified populations. However, this can be done adeptly by considering the advantage of laboratory research data, clinical trials, and observational studies that employ a different range of available scientific methods to establish evidence that is presented in our conceptual model, shown in Fig. 18.1.

As per our conceptual model, population data is detailed studies of human with impact of physical, mental, socio-economical, and regional and ethnic effect. Based on this multi-disciplinary impact, data available on humans particularly fall into two categories: data related to epidemiology, which reveal patterns of disease rate and mortality rate in different or single groups that were exposed to variety of toxic substances and the clinical data of affected population that have exposed to these toxic agents (Page et al. 1976). In investigating the occurrence of specific type of cancer in a population, epidemiologists often begin by describing the type of cancer and its related or non-related conditions that previous knowledge suggests may be related to it. The knowledge concerning the effect of toxic materials on human health has come from clinical data after exposure to high dose of carcinogen and industrial accidents. The report of exposure to carcinogen has typically identified acute effects but continued exposure can also lead to chronic and delayed onset of tumor formation (Perera and Weinstein 1982). The pathogenesis of any type of cancer in large population at shorter duration of time, is potentially due to regular and continued exposure of potential carcinogen, which might be due to consumption of water source having carcinogen associated waste, working area or eruption of harmful industrial gaseous waste. The main delay in finding the risk factor is long

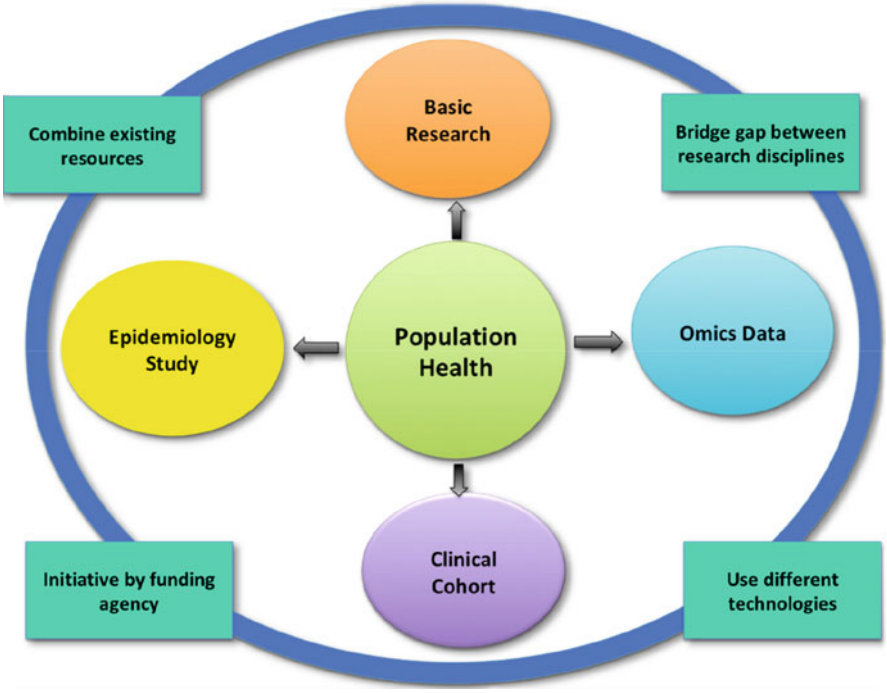


Fig. 18.1 Translational epidemiology model

silent asymptomatic periods, difficulties in making quantitative estimates of exposure of potential carcinogen, and problems in conducting follow-up studies of affected populations after exposure, epidemiological data of these exposed populations are unlikely to be available for most carcinogens and particularly most of carcinogens agents were found in drinking water until many years after first exposures (Calabrese 1983; Cantor et al. 1985; Harrington et al. 1978). As per our conceptual model, collecting population of data of such populations is very challenging. So, it's imperative to formulate certain guidelines of collecting and managing clinical, demographic and biochemical data of such exposed populations suffering from cancer. This problem could also be addressed by funding agencies to start with initiative to foster research in this area, which directly helps to collect and manage the population data.

The new information uncovered by the basic research in different areas of cancer plays a huge role in success of translational epidemiology. So, the main aim of translational epidemiology is to address all the associated factor that could prevent the development of cancer, early diagnosis of pre-malignant stage and treatment regime. Many cancers can be treated by localized treatments that include surgical intervention or by the radiation, if they are detected early or before metastasis. Only minor surgical intervention is required for the complete cure of the early premalignant stages of colon adenomas. Particularly, in case of early carcinomas, that remain

localized to their site of origin; they can be easily cured with the cure rate of 90%. But the survival rate would drop about 50% in those patients whose cancers have almost spread to adjacent organs and lymph nodes, and the survival rate is less than 10% for patients, which have metastatic colon cancer (O'Connell et al. 2004). This pattern of survival rate is predominately have witnesses in almost all type of solid tumor and cancers. So, based on these findings, early detection of specific type of cancers can thus be a condemnatory determinant of the result of the disease. The role of basic science research is to design the preventive measure and early diagnosis, which are majorly based on the identification of subject with inherited susceptibilities or genetic predisposition to development of cancer. These types of inherited cancer susceptibilities can be due to different pathogenic mutations in important tumor suppressor genes or in at least one oncogene or proto-oncogene, in genes responsible for DNA repair genes, such as the mismatch repair genes that are main contributing factor for the development of hereditary non polyposis colon cancer.

With advent of technologies like next-generation sequencing, pyro sequencing, construction of sophisticated online or application based algorithms, and software for omics data analysis, multiple patients of different types of cancer can be analyzed in single platform. This will uncover the germ line as well as somatic rare mutations in multiple genes that allow the identification and stratification of individuals that have high-risk of developing disease in future. These technologies, like next generational sequencing plays predominant role in translational epidemiology as it has ability to produce high throughout data and also saves tremendous amount of time. The mutation data of disease causing genes, risk associated genes as well as mutation pattern in modifies genes, so bio-informatics data related to mutations associated with particular group of cancer can be correlated with the demographic, clinical, bio clinical data of specific cancer type or related cancer type can be done in order to identify the cancer diagnosis and prediction marker. So, it's very imperative to combine all the aspects of epidemiology with the basic research to identify specific biomarker for the diagnosis of cancer. Figure 18.1 demonstrates the hypothetical model of translational epidemiology in which there is culmination of basic research, clinical cohort data, epidemiology data, and population data along with the financial support from different funding agency.

Further the data related to patient history can also pose a preventive option of cancer. For example, in case of colon adenoma/cancer, they can be detected by the colonoscopy and the tumor part can be removed prior to the development of secondary organ malignancy. The germ line and inherited mutation of tumor suppressor gene, *APC* gene occurs in subjects with familial adenomatous polyposis. The individual having germ line mutation in *APC* gene are likely to transform into hundreds of adenomas within the first 20 years of life, so the colons of these susceptible subjects were preferably removed before the progression of these pre-metastatic polyps to malignancy (Fodde 2002). So, the careful monitoring of patients history, recording patients and its family member data along with systematic analysis of its associated basic research data (omics and histopathological analysis) can lead to fruitful road in cancer diagnosis as well as designing potential treatment

regime. In this aspect, translational epidemiology in the field of cancer should be implicated into general research practice with proper guidelines that can provide successful translational outcome.

References

Abernethy AP, Etheredge LM, Ganz PA, Wallace P, German RR, Neti C, Bach PB, Murphy SB (2010) Rapid-learning system for cancer care. J Clin Oncol 28(27):4268

Augustin HG (2003) Translating angiogenesis research into the clinic: the challenges ahead. Br J Radiol 76:S3–S10

Azad NS, Rasool N, Annunziata CM, Minasian L, Whiteley G, Kohn EC (2006) Proteomics in clinical trials and practice; present uses and future promise. Mol Cell Proteomics 5:1819–1829

Beasley JM, Kwan ML, Chen WY, Weltzien EK, Kroenke CH, Lu W, Nechuta SJ, Cadmus-Bertram L, Patterson RE, Sternfeld B, Shu XO (2012) Meeting the physical activity guidelines and survival after breast cancer: findings from the after breast cancer pooling project. Breast Cancer Res Treat 131(2):637–643

Best T, Li D, Skol AD, Kirchhoff T, Jackson SA, Yasui Y, Bhatia S, Strong LC, Domchek SM, Nathanson KL, Olopade OI (2011) Variants at 6q21 implicate PRDM1 in the etiology of therapy-induced second malignancies after Hodgkin's lymphoma. Nat Med 17(8):941–943

Borrebaeck CA (2017) Precision diagnostics: moving towards protein biomarker signatures of clinical utility in cancer. Nat Rev Cancer 17(3):199–204

Burgio MR, Ioannidis JP, Kaminski BM, DeRycke E, Rogers S, Khoury MJ, Seminara D (2013) Collaborative cancer epidemiology in the 21st century: the model of cancer consortia. Cancer Epidemiol Biomarkers Prev 22(12):2148–2160

Caan BJ, Natarajan L, Parker B, Gold EB, Thomson C, Newman V, Rock CL, Pu M, Al-Delaimy W, Pierce JP (2011) Soy food consumption and breast cancer prognosis. Cancer Epidemiol Biomarkers Prev 20(5):854–858

Calabrese EJ (1983) Role of epidemiologic studies in deriving drinking water standards for metals. Environ Health Perspect 52:99–106

Cameron D, Jones IG (1983) John Snow, the Broad Street pump and modern epidemiology. Int J Epidemiol 12(4):393–396

Cantor KP, Hoover R, Hartge P, Mason TJ, Silverman DT, Levin LI (1985) Drinking water source and risk of bladder cancer: a case-control study. In: Jolley RL, Bull RJ, Davis WP, Katz S Jr, Roberts MH, Jacobs VA (eds) Water chlorination: chemistry, environmental impact and health effects, vol 5. Lewis Publishers, Chelsea, pp 143–150

Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, Douville C, Javed AA, Wong F, Mattox A, Hruban RH (2018) Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 359(6378):926–930

Cronin M, Sangli C, Liu ML et al (2007) Analytical validation of the Oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response prediction in node-negative, estrogen receptor-positive breast cancer. Clin Chem 53(6):1084–1091

Dagogo-Jack I, Shaw AT (2018) Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol 15(2):81

de González AB, Morton LM (2012) Converting epidemiologic studies of cancer etiology to survivorship studies: approaches and challenges. Cancer Epidemiol Biomarkers Prev 21(6): 875–880

Fodde R (2002) The APC gene in colorectal cancer. Eur J Cancer 38(7):867–871

Freedman AN, Sansbury LB, Figg WD, Potosky AL, Weiss Smith SR, Khoury MJ et al (2010a) Cancer pharmacogenomics and pharmacoepidemiology: setting a research agenda to accelerate translation. J Natl Cancer Inst 102:1698–1705

Freedman JE, Larson MG, Tanriverdi K, O'Donnell CJ, Morin K, Hakanson AS, Vasan RS, Johnson AD, Iafrati MD, Benjamin EJ (2010b) The relation of platelet and leukocyte inflammatory transcripts to body mass index in the Framingham heart study. Circulation 122(2):119

Freedman JE, Gerstein M, Mick E, Rozowsky J, Levy D, Kitchen R, Das S, Shah R, Danielson K, Beaulieu L, Navarro FC (2016) Diverse human extracellular RNAs are widely detected in human plasma. Nat Commun 7(1):1–4

Frueh FW (2009) Back to the future: why randomized controlled trials cannot be the answer to pharmacogenomics and personalized medicine. Pharmacogenomics 10(7):1077–1081

Fu Z, Zhang R, Li P, Jia M (2019) Translational epidemiology: the powerful tool for precision cancer medicine. J Cancer Res Ther 15(2):269

Garraway LA (2013) Genomics-driven oncology: framework for an emerging paradigm. J Clin Oncol 31(15):1806–1814

Hall KL, Feng AX, Moser RP, Stokols D, Taylor BK (2008) Moving the science of team science forward: collaboration and creativity. Am J Prev Med 35(2):S243–S249

Hall KL, Stokols D, Stipelman BA, Vogel AL, Feng A, Masimore B, Morgan G, Moser RP, Marcus SE, Berrigan D (2012) Assessing the value of team science: a study comparing center-and investigator-initiated grants. Am J Prev Med 42(2):157–163

Harrington JM, Middaugh JP, Morse DL, Housworth J (1978) A survey of a population exposed to high concentrations of arsenic in well water in Fairbanks, Alaska. Am J Epidemiol 108:377–385

Hermiston TW, Kirn DH (2005) Genetically based therapeutics for cancer: similarities and contrasts with traditional drug discovery and development. Mol Ther 11:496–507

Hudson MM, Mertens AC, Yasui Y, Hobbie W, Chen H, Gurney JG, Yeazel M, Recklitis CJ, Marina N, Robison LR, Oeffinger KC (2003) Health status of adult long-term survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. JAMA 290(12): 1583–1592

Huguet F, Giocanti N, Hennequin C, Croisy M, Touboul E, Favaudon V (2008) Growth inhibition by STI571 in combination with radiation in human chronic myelogenous leukemia K562 cells. Mol Cancer Ther 7:398–406

Ioannidis JP, Schully SD, Lam TK, Khoury MJ (2013) Knowledge integration in cancer: current landscape and future prospects. Cancer Epidemiol Biomarkers Prev 22(1):3–10

Karagoz K, Sinha R, Arga KY (2015) Triple negative breast cancer: a multi-omics network discovery strategy for candidate targets and driving pathways. OMICS 19(2):115–130

Karczewski KJ, Snyder MP (2018) Integrative omics for health and disease. Nat Rev Genet 19(5): 299

Kessel F, Norman Anderson American Psychological Association (eds) (2008) Interdisciplinary research: case studies from health and social science. Oxford University Press, Oxford

Khoury MJ, Gwinn M, Yoon PW, Dowling N, Moore CA, Bradley L (2007) The continuum of translation research in genomic medicine: how can we accelerate the appropriate integration of human genome discoveries into health care and disease prevention? Genet Med 9(10):665–674

Khoury MJ, Gwinn M, Ioannidis JP (2010) The emergence of translational epidemiology: from scientific discovery to population health impact. Am J Epidemiol 172(5):517–524

Khoury MJ, Gwinn M, Dotson WD, Schully SD (2012) Knowledge integration at the center of genomic medicine. Genet Med 14(7):643–647

Kris MG, Johnson BE, Berry LD, Kwiatkowski DJ, Iafrate AJ, Wistuba II, Varella-Garcia M, Franklin WA, Aronson SL, Su PF, Shyr Y (2014) Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. JAMA 311(19):1998–2006

Kwan ML, Chen WY, Kroenke CH, Weltzien EK, Beasley JM, Nechuta SJ, Poole EM, Lu W, Holmes MD, Quesenberry CP, Pierce JP (2012) Pre-diagnosis body mass index and survival after breast cancer in the After Breast Cancer Pooling Project. Breast Cancer Res Treat 132(2): 729–739

Lam TK, Spitz M, Schully SD, Khoury MJ (2013) "Drivers" of translational cancer epidemiology in the 21st century: needs and opportunities. Cancer Epidemiol Biomarkers Prev 22(2):181–188

Lau B, Duggal P, Ehrhardt S, Armenian H, Branas CC, Colditz GA, Fox MP, Hawes SE, He J, Hofman A, Keyes K (2020) Perspectives on the future of epidemiology: a framework for training. Am J Epidemiol 189(7):634–639

Li S (2008) Src-family kinases in the development and therapy of Philadelphia chromosome-positive chronic myeloid leukemia and acute lymphoblastic leukemia. Leuk Lymphoma 49: 19–26

Lilienfeld DE (2000) John Snow: the first hired gun? Am J Epidemiol 152(1):4–9

Little J, Sharp L, Khoury MJ, Bradley L, Gwinn M (2005) The epidemiologic approach to pharmacogenomics. Am J Pharmacogenomics 5(1):1–20

Madlensky L, Natarajan L, Tchu S, Pu M, Mortimer J, Flatt SW, Nikoloff DM, Hillman G, Fontecha MR, Lawrence HJ, Parker BA (2011) Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. Clin Pharmacol Ther 89(5):718–725

Nechuta SJ, Caan BJ, Chen WY, Flatt SW, Lu W, Patterson RE, Poole EM, Kwan ML, Chen Z, Weltzien E, Pierce JP (2011) The After Breast Cancer Pooling Project: rationale, methodology, and breast cancer survivor characteristics. Cancer Causes Control 22(9):1319–1331

Nelson HD, Huffman LH, Fu R, Harris EL (2005) Genetic risk assessment and BRCA mutation testing for breast and ovarian cancer susceptibility: systematic evidence review for the US Preventive Services Task Force. Ann Intern Med 143(5):362–379

Nhung NT, Khuong VT, Huy VQ (2016) Classifying prostate cancer patients based on total prostate-specific antigen and free prostate-specific antigen features by support vector machine. J Cancer Res Ther 12(2):818

O'Connell JB, Maggard MA, Ko CY (2004) Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst 96(19):1420–1425

Oeffinger KC, Mertens AC, Sklar CA, Kawashima T, Hudson MM, Meadows AT, Friedman DL, Marina N, Hobbie W, Kadan-Lottick NS, Schwartz CL (2006) Chronic health conditions in adult survivors of childhood cancer. N Engl J Med 355(15):1572–1582

Ogino S, King EE, Beck AH, Sherman ME, Milner DA, Giovannucci E (2012) Interdisciplinary education to integrate pathology and epidemiology: towards molecular and population-level health science. Am J Epidemiol 176(8):659–667

Omenn GS, Nass SJ, Micheel CM (eds) (2012) Evolution of translational omics: lessons learned and the path forward. National Academies Press

Page T, Harris RH, Epstein SS (1976) Drinking water and cancer mortality in Louisiana. Science 193:55–57

Perera FP, Weinstein IB (1982) Molecular epidemiology and carcinogen-DNA adduct detection: new approaches to studies of human cancer causation. J Chronic Dis 35:581–600

Petricoin EF III, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA (2002a) Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359(9306):572–577

Petricoin EF III, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, Velassco A, Trucco C, Wiegand L, Wood K, Simone CB (2002b) Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 94(20):1576–1578

Pierce JP, Natarajan L, Caan BJ, Parker BA, Greenberg ER, Flatt SW, Rock CL, Kealey S, Al-Delaimy WK, Bardwell WA, Carlson RW (2007) Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: the Women's Healthy Eating and Living (WHEL) randomized trial. JAMA 298(3):289–298

Rastogi T, Hildesheim A, Sinha R (2004) Opportunities for cancer epidemiology in developing countries. Nat Rev Cancer 4(11):909–917

Robison LL, Armstrong GT, Boice JD, Chow EJ, Davies SM, Donaldson SS, Green DM, Hammond S, Meadows AT, Mertens AC, Mulvihill JJ (2009) The Childhood Cancer Survivor Study: a National Cancer Institute–supported resource for outcome and intervention research. J Clin Oncol 27(14):2308

Rosenfield PL (1992) The potential of transdisciplinary research for sustaining and extending linkages between the health and social sciences. Soc Sci Med 35(11):1343–1357

Saijo N, Nishio K, Tamura T (2003) Translational and clinical studies of target-based cancer therapy. Int J Clin Oncol 8:187–192

Sellers TA (2006) The beginning of the end for the epidemiologic focus on gene-environment interactions? Cancer Epidemiol Biomark Prev 15:1059–1060

Shah R, Pico AR, Freedman JE (2016) Translational epidemiology: entering a brave new world of team science. Circ Res 119(10):1060–1062

Shpilberg O, Dorman JS, Ferrell RE, Trucco M, Shahar A, Kuller LH (1997) The next stage: molecular epidemiology. J Clin Epidemiol 50(6):633–638

Shrager J, Tenenbaum JM (2014) Rapid learning for precision oncology. Nat Rev Clin Oncol 11(2): 109

Singer DS, Jacks T, Jaffee E (2016) A US "Cancer Moonshot" to accelerate cancer research. Science 353(6304):1105–1106

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235(4785):177–182

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244(4905):707–712

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344(11):783–792

Sleijfer S, Bogaerts J, Siu LL (2013) Designing transformative clinical trials in the cancer genome era. J Clin Oncol 31(15):1834–1841

Smith GD (2010) Mendelian randomization for strengthening causal inference in observational studies: application to gene × environment interactions. Perspect Psychol Sci 5(5):527–545

Sondka Z, Bamford S, Cole CG et al (2018) The COSMIC cancer gene census: describing genetic dysfunction across all human cancers. Nat Rev Cancer 18(11):696–705

Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P, Børresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. PNAS 98:10869–10874

Spitz MR, Caporaso NE, Sellers TA (2012) Integrative cancer epidemiology—the next generation. Cancer Discov 2(12):1087–1090

Sung NS, Crowley WF Jr, Genel M, Salber P, Sandy L, Sherwood LM, Johnson SB, Catanese V, Tilson H, Getz K, Larson EL (2003) Central challenges facing the national clinical research enterprise. JAMA 289(10):1278–1287

Thomas DC, Conti DV (2004) Commentary: the concept of 'Mendelian Randomization'. Int J Epidemiol 33(1):21–25

Thun MJ, Hoover RN, Hunter DJ (2012) Bigger, better, sooner–scaling up for success. Cancer Epidemiol Biomarkers Prev 21(4):571–575

van 't Veer LJ, Dai H, van de Vijver MJ et al (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415(6871):530–536

Verma M, Khoury MJ, Ioannidis JP (2013) Opportunities and challenges for selected emerging technologies in cancer epidemiology: mitochondrial, epigenomic, metabolomic, and telomerase profiling. Cancer Epidemiol Biomarkers Prev 22(2):189–200

Wang F, Sc W, Chan LW et al (2014) Multiple regression analysis of mRNA-miRNA associations in colorectal cancer pathway. Biomed Res Int 2014:676724

Westfall JM, Mold J, Fagnan L (2007) Practice-based research—"blue highways" on the NIH roadmap. JAMA 297(4):403–406

Woolf SH (2008) The meaning of translational research and why it matters. JAMA 299(2):211–213

Wright C, Angus B, Nicholson S, Sainsbury JR, Cairns J, Gullick WJ, Kelly P, Harris AL, Horne CW (1989) Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Res 49(8):2087–2090

Xiao L, Xiao T, Wang ZM et al (2014) Biomarker discovery of nasopharyngeal carcinoma by proteomics. Expert Rev Proteomics 11(2):215–225

Adenosine Pathway in Genitourinary Malignancies: A Promising Immunotherapeutic Target

19

Sumit Dey and Ravimohan S. Mavuduru

Abstract

Pathways involved in adenosine metabolism have emerged as an exciting drug target in different types of cancers. Mechanistically it is proposed that there is a rapid increase in adenosine metabolism within the tumour microenvironment, which in turn suppress the immune-mediated tumour cell killing. Adenosine converting enzymes CD39 and CD73 and extracellular adenosine receptors A1, A2, A3 and A4 are regarded as the important regulators involved. Their expression in cancer cells are seen to be related to the tumour growth. Genitourinary cancer comprises of heterogenous group of tumours with different physiological function and nature of the organ system. During the last 5 years, new age immunotherapeutic drugs have revolutionized the treatment of different types of genitourinary cancers. Signalling components of adenosine pathway are thus promising since they, too, essentially modulate the immunotherapeutic targets. This review summarizes the literature on anticancer immunotherapeutic effect of adenosine pathway in genitourinary cancer. At first the mechanisms through which adenosine pathway exerts its immunosuppressive effect in different cancers are discussed. Thereafter, the preclinical and clinical validation of adenosine therapy in different types of genitourinary cancer are discussed. Subsequent to this, various ongoing multicancer clinical trials on the experimental drugs, targeting adenosine metabolism in genitourinary cancers, are tabulated with some perspective on the future research.

Keywords

Adenosine · Tumour microenvironment · T cell · Macrophage · Natural killer cells · Genitourinary cancer · Immunotherapy · Clinical trial

S. Dey · R. S. Mavuduru (✉)
Department of Urology, PGIMER, Chandigarh, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_19

19.1 Background: Adenosine and Its Possible Link to Cancer and Genitourinary Cancer in Particular

Adenosine, an organic compound is one of the four nucleoside building blocks of RNA (Boyle 2005). It is the extracellular adenosine which is the matter of interest. Concentration of extracellular adenosine varies depending on the abnormal pathophysiological conditions such as hypoxia, ischaemia, inflammation, or trauma (Allard et al. 2020a). Chu et al. in 1975 for the first time, demonstrated that extracellular adenosine (eADO) is responsible for cytotoxic T cell mediated immunosuppression against EL4 lymphoma cells in vitro in association with increased intracellular levels of cyclic AMP (cAMP) (Wolberg et al. 1975). Interestingly, upon its release into the extracellular space, adenosine forwards a danger signal to the surrounding environment and activates an array of signalling cascade. High extracellular adenosine concentration concomitantly activates adenosine receptors present on target cells, thereby activating various cellular responses to restore tissue homeostasis (Allard et al. 2020a; Chen et al. 2013). During acute injury, eADO concentration increases in the extracellular milieu, and this further exerts protective effects by shielding cells and tissues from an excessive inflammatory response and immune-mediated damage (Antonioli et al. 2019).

Persistent increased levels of adenosine promote cellular remodelling by activating immune suppressive pathways and inhibiting natural wound healing process (Haskó et al. 2008). These signalling changes further induce continuous cell proliferation and neoplastic transformation. Chronic accumulation of adenosine in the tumour microenvironment has been associated with the generation of an immunosuppressed niche favouring cell proliferation, angiogenesis through multiple mechanisms including inhibition of T helper 1 cell (TH1 cell) mediated cytokine production, deregulation of mononuclear phagocyte cell differentiation and maturation, and suppression of effector T cells (Allard et al. 2020a; Boison and Yegutkin 2019; Vigano et al. 2019). In addition to the effects of adenosine on the cancer microenvironment, which indirectly affect the course of cancer development, progression and metastasis are also determined by the direct effects of adenosine on cancer cells (Ghiringhelli et al. 2012). Thus, adenosine is believed to be a crucial regulatory molecule acting both as an autocrine as well as paracrine factor inside the tumour microenvironment (Ohta et al. 2006; Blay et al. 1997).

Genitourinary (GU) malignancies comprise of diverse groups of solid cancers completely different from each other in anatomy and function. As a matter of fact, data suggests that metastatic genitourinary cancer causes an annual morbidity of 225,000 patients with mortality of over 56,000 patients per year in the USA (Siegel et al. 2019). Recently, research to find out new therapeutic targets in genitourinary cancers have gained momentum after the promising results from immunotherapeutic drugs in these group of cancers (Zarrabi et al. 2019). Adenosine signalling has been at the forefront of immunosuppression in cancer cells, and recent preclinical studies targeting different adenosine receptors and enzymes have shown exciting results in different genitourinary cancers. There are at least 18–20 ongoing clinical trials with experimental drugs targeting adenosinergic signalling system against different solid

cancers including genitourinary cancers. Two of these trials have published encouraging preliminary findings on metastatic castration resistance prostate cancer (Harshman et al. 2020) and treatment refractory renal cell cancer (Fong et al. 2020). Thus clearly eADO has a role which needs to be understood and explored.

19.2 Adenosine Signalling

To understand further, let us first take a brief look at the mechanism of eADO production and concomitant signalling pathways involved with adenosine. Extracellular adenosine generation, degradation, recycle and biological use is governed by purinergic pathways comprising of a diverse group of enzymes and related transporters. Production of eADO is mediated mostly by two main pathways: canonical and non-canonical generation of eADO (Ferretti et al. 2018).

(a) **Canonical Pathway**: This pathway is governed by enzymes ectonucleotidases CD39 (ectonucleoside triphosphate diphosphohydrolase) and CD73 (5-′-nucleotidase) which hydrolyses the extracellular ATP (Yegutkin et al. 2002). In short, CD39 is a transmembrane enzyme (Kaczmarek et al. 1996) that converts extracellular ATP to ADP and AMP. This extracellular AMP generated by CD39 gets converted to extracellular adenosine (eADO) by another glycophosphatidylinositol-anchored (GPI) enzyme CD73 (Zimmermann 1992). Regulation of extracellular AMP (eAMP) concentration is further mediated by membrane associated forms of adenylate kinase (ecto-AK) and nucleoside diphosphate kinase (ecto-NDPK) that phosphorylate eAMP to generate eATP (Donaldson et al. 2002).

(b) **Non-canonical Pathway**: In this, eAMP is generated using NAD^+ as a substrate by CD38 and CD203a. CD38 is an NAD^+ dependent ectohydrolase, also known as ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1 and CD203a is otherwise known as ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) or PC-1 (Deterre et al. 1996; Horenstein et al. 2013). Next, CD73 hydrolyses the eAMP produced by this process. Simultaneously, tissue-specific alkaline phosphatases like prostatic acid phosphatase and tissue-non-specific alkaline phosphatases (TNAPs) can also hydrolyse eAMP to eADO (Street et al. 2013).

19.2.1 Components of Adenosine Signalling

19.2.1.1 Adenosine Receptors

These are eADO-specific receptors also known as P1 receptors, consisting of four distinct G protein-coupled receptors: A1, A2A, A2B and A3. Human P1 receptors A1, A2A and A3 have varying affinity for eADO which falls in the nanomolar range (Müller and Jacobson 2011). Physiological and steady state concentration of eADO also corresponds to this range (Fredholm 2007). By contrast, under pathological

conditions, when eADO level increases, A2B receptor is activated (Fredholm et al. 2011). A2B is a low-affinity receptor that has an affinity for eADO in micromolar range (Kd of 15 μM), which usually only occurs under pathological conditions (Müller and Jacobson 2011; Fredholm et al. 2011). P1 receptors modulate adenylate cyclase activity thereby balancing the intracellular level of cAMP and in turn level of eADO. A1 and A3 are Gi/o-coupled adenosine receptors which inhibit the adenylate cyclase activity which in turn decreases intracellular cAMP level (Merighi et al. 2018). On the other hand, A2A and A2B receptors are Gs-coupled receptor, and they activate adenylate cyclase and trigger cAMP dependent downstream signalling event such as stimulation of cAMP-PKA response element binding protein (CREBP) pathway. However, eADO receptors can also work through cAMP-independent pathways. A2B and A3 may act through phospholipase C (PLC) → inositol 1,4,5-trisphosphate (IP_3) → Ca2+ and/or phospholipase C → diacylglycerol (DAG) → PKC pathways (PLC/PI_3/DAG). Moreover, A2A and A2B can also activate the ERK and/or p38 MAPK and PI3K–AKT–mTOR pathways (Allard et al. 2020a; Merighi et al. 2018).

19.2.1.2 Adenosine Converting Enzymes: CD39 and CD73

Dying and stressed cells release ATP which in turn provide inflammatory signals crucial for effective innate and adaptive immune responses. Conversely, hydrolysis of extracellular ATP into adenosine serves to limit immune response. CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD-1)) is the rate-limiting ecto-enzyme in the hydrolysis of extracellular adenosine triphosphate (ATP) (Allard et al. 2020b).

Under normal conditions, extracellular ATP concentration ranges from 10 to 100 nM. However, its concentration rapidly increases in response to tissue injury and hypoxia, and also can be found at high concentrations in tumours (1–50 μM) (Feng et al. 2020). These increased ATP concentration inside tumour microenvironment is constantly converted to adenosine by CD39-CD73 enzyme system accumulating more adenosine inside (Allard et al. 2020b; Feng et al. 2020). Increased adenosine in turn modulate diverse signalling cascades including immune system. According to Mosser and colleagues, CD39 acts as a 'molecular switch' that controls the balance between inflammatory and regulatory macrophage differentiation (Cohen et al. 2013). Increasing extracellular ATP levels also promote macrophage phagocytosis through calcium-mediated autocrine and paracrine signalling (Zumerle et al. 2019). Tumour-specific CD8+ T cells found in human tumours express high levels of CD39, are enriched for T cells against neoantigens and exhibit an exhausted gene signature (Simoni et al. 2018). CD39+ CD8+ T cells were found to be enriched within the TME, invaded lymph nodes and metastases compared with healthy tissues (Duhen et al. 2018; Canale et al. 2018). In similar way, CD39 acts upon NK cells, dendritic cells and regulatory T cells which together with other signalling system help tumour cells evade immune cell mediated killing (Allard et al. 2020b).

While CD39 degrades ATP to produce adenosine monophosphate (AMP), CD73 converts AMP to immunosuppressive adenosine (Allard et al. 2020a). CD73 is also

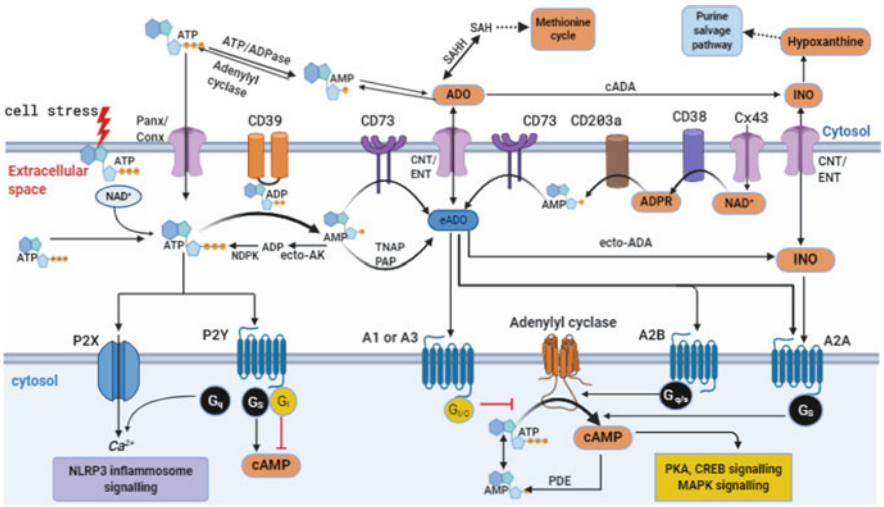


Fig. 19.1 Schematic illustration of eADO production, regulation, clearance and signalling: Channel proteins—connexin (Conx) and pannexin (Panx). Purinergic receptors—P2X and P2Y. *NLRP3* NOD-, LRR- and pyrin domain-containing protein 3. *ecto-AK* extracellular adenylate kinase. *NDPK* nucleoside diphosphate kinase. *ADPR* ADP-ribose. *CD203a/ENPP1* ectonucleotide pyrophosphatase/phosphodiesterase family member 1. Type 1 purinergic (P1) receptors—A1, A2A, A2B and A3. Inosine- INO- inosine. *ecto-ADA* adenosine deaminase. *ENTs and CNTs* nucleoside transporters. *cADA* cytosolic form of adenosine deaminase. *SAH S*-adenosyl-homocysteine, *SAHH S*-adenosyl-homocysteine hydrolase, *ADK* adenosine kinase, Intracellular ATP can be subsequently generated from AMP and ADP via adenylate kinases, *PDE* phosphodiesterase, The eADO receptors A1, A2A, A2B and A3 can upregulate or downregulate adenylate cyclase activity and, thus, the initiation of cAMP-dependent signalling. CREB-cAMP-responsive element-binding protein 1, Cx43-connexin 43

detected as a soluble form (sCD73) in the plasma of cancer patients (Morello et al. 2017). In addition to hypoxia, several factors including growth factor independent 1 (Gfi1), signal transducer and activator of transcription 3 (STAT3), Sp1, IL-6, interferon (IFN) type I, TGF-β1, IL-1β, TNF-α, prostaglandin E2, Wnt signalling and protein kinase C (PKC) can induce expression of CD73 (Jadidi-Niaragh 2019). In contrast, some factors such as IL-12, IL-21, IL-4 and IFN-γ suppress CD73 expression (Ghalamfarsa et al. 2019). Several preclinical results suggested that CD39-CD73 axis can be a promising immunotherapeutic target against solid cancers. The role of CD39-CD73 signalling system in genitourinary cancer will be discussed more in detail in later part of this review (Fig. 19.1).

19.2.2 Adenosine Signalling and Cancer

Hypoxic environment influences solid tumour growth which in turn induces accumulation of eADO (Vaupel and Mayer 2016). Hypoxia induces overexpression of transcription factor HIF1, subsequently triggering expression of CD39 and CD73 on

variety of cells in tumour microenvironment such as stromal cells, tumour infiltrating immunosuppressive cell subsets like regulatory T cells (Treg), and myeloid derived suppressor cells (MDSC) (Boison and Yegutkin 2019). Mice deficient in CD39 or CD73 are susceptible to inflammation and resistant to tumour growth as well due to alleviation of eADO-mediated immunosuppression. CD38, a non-canonical eADO generating ectohydrolase is highly expressed on tumour stromal cells, T cell subset, and NK cells which further stimulate suppression of T cell function and proliferation (Allard et al. 2017).

19.2.2.1 Effect on Cancer Immunity

Suppression of T Cell Mediated Tumour Inhibition

Elevated adenosine level in tumour microenvironment induces intracellular cAMP accumulation thereby impairing T-cell function. In general, T cell surface expresses all the four adenosine receptors (Vigano et al. 2019). More specifically, A2A receptor upregulation inside T cell subpopulation further leads to the imbalance, thereby promoting intracellular cAMP build up inside tumour microenvironment which may activate myriad of connecting cell survival and proliferation pathways (Sek et al. 2018).

Inhibitory Effect in Tumour Microenvironment (TME)

Tumour microenvironment is composed of varied cell types, which plays an important role in tumour proliferation and resistance to immune cell mediated killing (Whiteside 2008). These cell types also express ARs (Arab and Hadjati 2019). These purinergic receptors sense the accumulation of adenosine inside the TME and help them achieve the immune resistance of cancer cells (Whiteside 2008; Arab and Hadjati 2019).

Dendritic Cells (DC)

Dendritic cells are important connecting link between the innate and adaptive immune system, and their role can be severely impaired by adenosinergic signalling (Vigano et al. 2019). It has been reported that adenosine binding to A_2B receptor impairs the differentiation process of dendritic cells from monocytes (Novitskiy et al. 2008; Challier et al. 2013). In another report, it was seen that adenosine mediated stimulation of A_2A and A_2B receptor diminishes the capacity of DCs to prime T-helper cell mediated adaptive immunity (Yang et al. 2010). Adenosine-treated DCs exhibited upregulation of PD-L2 (Li et al. 2012) due to high A_2A receptor expression, which further leads to immune resistance to cancer cells (Vigano et al. 2019).

Macrophages

Monocyte to Macrophage differentiation gets hindered by activation of adenosinergic signalling probably through cAMP accumulation (Najar et al. 1990). Moreover, adenosine reduces the pro-inflammatory activity of macrophages by dampening their ability to produce IL-12, TNFα, macrophage inflammatory

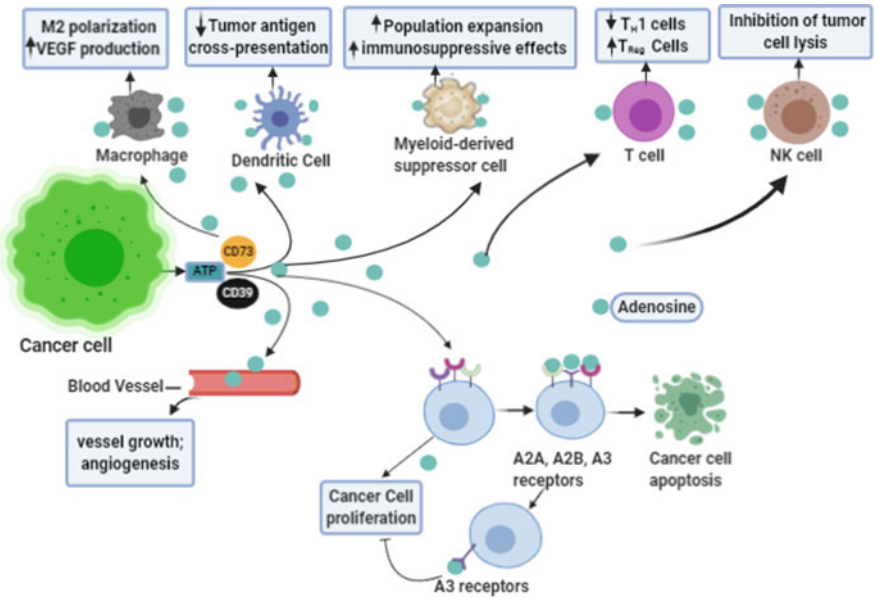


Fig. 19.2 Schematic illustration of adenosine mediated antitumor immunity

protein-1a (MIP1a), nitric oxide and superoxide (Haskó et al. 1996, 2000; Haschemi et al. 2007; Szabó et al. 1998).

Natural Killer Cells (NK Cells)

Adenosine mediated stimulation of A2AR receptor restricts NK cell maturation, proliferation and IFN-gamma and TNF-α production (Young et al. 2018; Miller et al. 1999; Raskovalova et al. 2006). Furthermore, A2AR receptor upregulation diminishes the ability of NK cells to targeted cell killing (Priebe et al. 1990) (Fig. 19.2).

19.3 Targeting Adenosine Receptor in Genitourinary Cancers

Genitourinary cancers, in particular carcinoma of the kidneys, bladder, and prostate take a large toll on human health and placed significant economic burden on health care systems (González del Alba et al. 2017). Prostate cancer ranks as the leading genitourinary cancer in the USA, followed by bladder and kidney cancer, which is the second most frequently diagnosed cancer and the sixth leading cause of cancer death among American males (Siegel et al. 2019). Renal cell carcinoma (RCC) accounts for approximately 2% of all types of cancers, which is growing annually at 1.5–5.9% around the world. Urinary bladder cancer is the ninth most common cancer in the world, with an estimated of 430,000 new cases diagnosed in 2012 (Cairns 2010). A better understanding of the biology of urologic malignancies has

Table 19.1 Expression of adenosine signalling enzymes and receptors in genitourinary cancer cell lines and tumour tissue. (↑—up regulation, ↓—down regulation)

GU cancer Expression profile	Renal cell carcinoma ↑ (up regulation) ↓ (down regulation)	Urothelial carcinoma ↑ (up regulation) ↓ (down regulation)	Prostate cancer ↑ (up regulation) ↓ (down regulation)
A1R	786-O, ACHN = ↑ (mRNA, protein) (Zhou et al. 2017a)	(↓-mRNA) 5637, EJ and T24 (cell line) (Zhou et al. 2017b)	(↑) slightly overexpressed than normal tissue (Mousavi et al. 2015)
A2bR	769-P (primary RCC), Caki-1 (metastatic RCC) = ↑ (mRNA, protein) (Yi et al. 2020)	(↑) (mRNA and protein) 5637, EJ and T24 Cell lines (Zhou et al. 2017b) (↑) (56/160) tumour tissue (Zhou et al. 2017b)	(↑) (mRNA and protein) PC-3, LNCaP, DU-145 cell lines (Mousavi et al. 2015) (↑) tumour tissue (significantly increased expression) (Mousavi et al. 2015)
A3R		(↑) T-24 cell line (A3aR) (Kim et al. 2010)	(↑) Moderately overexpressed in tumour tissue (Mousavi et al. 2015) (↑) overexpressed mRNA in PCa cell lines DU-145, PC3, LNCAp (Mousavi et al. 2015)
CD73/NT5E/ecto-5′-nucleotidase	(↑) 786-O cell line (stem cells) (↑) tumour tissue (Song et al. 2017)	(↑) (mRNA)-RT4, T24 cell lines (Stella et al. 2010) (↑) (MIBC) (↓) (NMIBC) (Wettstein et al. 2015; Dellê et al. 2020; Koivisto et al. 2019)	(↑) overexpression in tumour tissue (Leclerc et al. 2016) (↑) overexpressed in lymph node metastatic core (Yang et al. 2013)
CD39/NTPDase1/ Ectonucleoside triphosphate diphosphohydrolase-1	(↑) tumour tissue (Qi et al. 2020)	(↑) (NMIBC); (↓) (MIBC) (Dellê et al. 2020)	

led to a rapid change in their therapeutic landscape. With the approval of several novel agents in each of these tumours, understanding appropriate patient selection, mechanisms of resistance and optimal treatment sequence are critical components to improve patient outcome. Similarly, biomarker development is now a critical need in the field (Table 19.1).

Adenosine signalling pathway is now regarded as a hallmark for immune tolerance, which subsequently induces susceptibility of tumour cells to escape immune evasion (Sek et al. 2018). Its role in genitourinary cancer has recently caught interest and several ongoing clinical trials are underway to prove its effectiveness. Recent

advances in immune checkpoint inhibitors and its efficacy in GU cancers, mainly in urinary bladder (Pattanaik et al. 2019) and RCC have prompted researchers to study other major proteins involved in immune pathway regulation. In this section, we will highlight previous as well as ongoing work related to adenosine receptors and their agonists/antagonists in genitourinary cancer.

19.3.1 Preclinical and Clinical Results in GU Cancers

19.3.1.1 Renal Cell Carcinoma

Zhou et al. reported that in renal cancer cell line, adenosine A1 receptor expression is increased and pharmaceutical antagonist of A1R, DPCPX inhibit RCC cell proliferation through ERK/JNK signalling axis (Zhou et al. 2017a). Most recent study by **Yi et al**. proved that pharmacological blockade as well as shRNA mediated knockdown of A2b receptor reduced renal cancer cell proliferation, migration, invasion in vitro and tumour growth in vivo. Authors suggested that A2b receptor can be a potential anticancer target in RCC as A2b expression increases only in severe pathophysiological conditions, when extracellular adenosine release is higher as in the case of cancer cell proliferation and metastasis (Yi et al. 2020). VHL mutation is almost exclusive in RCC and in VHL-mutated RCC cell line; **Nagaya et al.** showed that adenosine A3 receptor actually helps RCC cells in apoptosis by upregulating AMID (AIF-homologous mitochondrion-associated inducer of death) expression (Nagaya et al. 2013). CD73 or ecto-5′-nucleosidase expression in RCC patients is negatively correlated with disease progression and overall survival rate. Significant association was observed in tumour type, tumour node metastasis (TNM) stage, and tumour grade (Yu et al. 2015). In RCC stem cells, CD73 was upregulated, which is highly tumorigenic in xenograft murine model (Song et al. 2017). In RCC microenvironment, tumour infiltrating T cytotoxic cells express high amount of CD39 which further aid in anticancer immunity in RCC cells (Qi et al. 2020) (Fig. 19.3).

19.3.1.2 Urothelial Cell Carcinoma

Phelps et al. reported that A1, A2a, and A2b are expressed in urinary bladder cancer cell line T24 but not A3 subtype. Further study with adenosine receptor specific ligands resulted in concentration dependent increase in intracellular Ca^{2+} ion with varied potency against different receptors (Phelps et al. 2006). **Cekic et al**., in the year 2011, showed that treatment of bladder and breast cancer cells with non-selective adenosine A_2B receptor antagonist both in vitro and in vivo slowed down the growth of both solid cancer types (Cekic et al. 2012). **Zhou et al**. showed that A2b receptor expression is upregulated in both bladder urothelial carcinoma tissue and cell line. A2bR expression pattern is inversely related to the disease progression. Further investigation revealed that A2bR mediated tumour progression is mediated by enhancement of MAPK signalling pathway (Zhou et al. 2017b). In a follow up study by the same group proved that treatment with the selective A2b receptor antagonist MRS1754 inhibited proliferation and migration of bladder

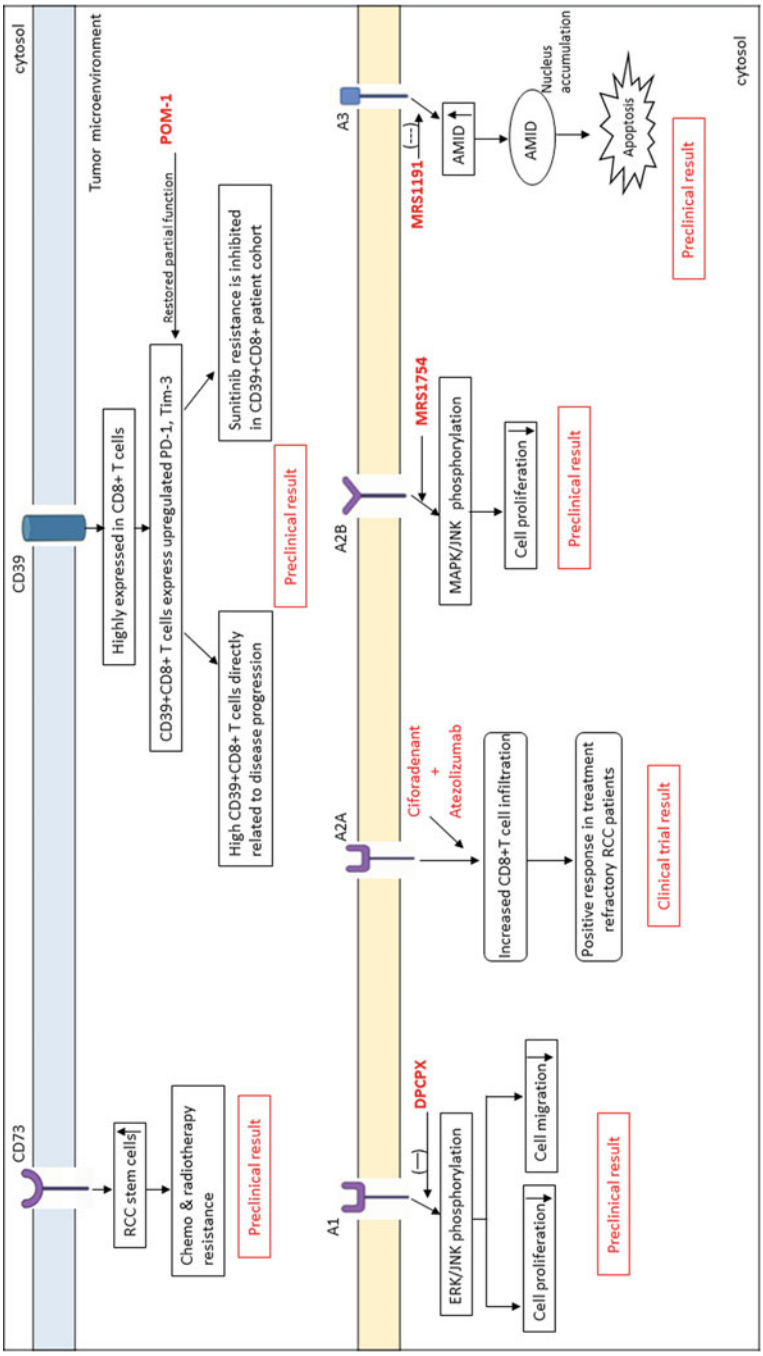


Fig. 19.3 Schematic representation of adenosine receptors, their antagonists and signalling counterparts in renal cell carcinoma

cancer cells by regulating MAP kinase pathway both in vivo and in vitro (Zhou et al. 2019). Most of the data on bladder cancer inhibition by adenosine receptors confirmed that adenosine A2b receptor can be a potential drug target in bladder cancer. However, in a 2012 study by **Kanno et al.** in 5637 bladder cancer cell line showed that adenosine A3 receptor induces apoptosis in bladder cancer cells via G_q/PKC (protein kinase C) mediated AIF (Apoptosis Inducing Factor) upregulation (Kanno et al. 2012). In a similar line of study, **Kim et al.** showed that treatment of bladder cancer cell line T24 by selective A3R agonist, truncated thio-CI-IB-MECA inhibited cancer cell proliferation, arrested cells at sub-G1 phase and induced apoptosis via ERK-JNK activation (Kim et al. 2010). **Hong-Li Cao et al.** showed that a natural bioactive compound cordycepin induces apoptosis in T24 bladder cancer cell line via activation of A3 adenosine receptor (Cao et al. 2017). On the other hand, inhibition of A2 adenosine receptor, which is upregulated in bladder cancer cells, induces cancer cell death. IHC analysis of non-muscle invasive bladder cancer (NMIBC) specimen revealed that another important enzyme in adenosine-signalling network, CD73/ecto-5′ nucleotidase, may serve as an excellent prognostic biomarker. High CD73 immunoreactivity was associated with favourable clinicopathological features. It also predicts better outcome in the subgroup of pTa and pT1 tumours (Wettstein et al. 2015). CD73 expression has been found to be indirectly related to radiation sensitivity in bladder cancer cell line in vitro (Dietrich et al. 2018). In a recent study by Koivisto et al. cell type specific expression of CD73 in urinary bladder cancer was assessed in both muscle invasive (MIBC) and non-muscle invasive (NMIBC). In log rank analysis, CD73 expression in bladder cancer cells was associated with better survival both in NMIBC and MIBC, whereas CD73 positivity in stromal fibroblasts was associated with impaired survival in NMIBC (Koivisto et al. 2019). In a recent interesting study, which is still in preprint version, ***Humberto Dellê* et al**. from Brazil showed that balanced expression between CD39 and CD73 can predict the invasiveness in human bladder cancer. Retrospective clinical follow-up data of urothelial bladder cancer specimens of 162 patients were collected, and immunohistochemistry for CD39 and CD73 was performed to make associations with clinicopathological data. High CD39 expression alone was more frequent in NMIBC ($p < 0.001$) type, while high expression of CD73 was more frequent in MIBC. When the authors combined the expression data of both CD39 and CD73 with respect to variants, it was found that association of both markers potentiated the prediction. High CD39 expression and low CD73 expression was strongly associated with NMIBC, while low CD39 with high CD73 was associated with MIBC (Dellê et al. 2020) (Fig. 19.4).

19.3.1.3 Prostate Cancer (PCa)

Prostate cancer progression follows multifaceted mechanism of action. **Mousavi et al**. reported that AR expression increases in malignant tumours and PCa cell lines (PC3, DU 145 and LNCaP) compared to normal tissues. Interestingly, there was no significant differences between A1 adenosine receptor expression in malignant and normal cells. This confirms that AR up-regulation in PCa tissue is in line with the upregulated adenosine receptor expression in other solid cancers. Overall, A3 and

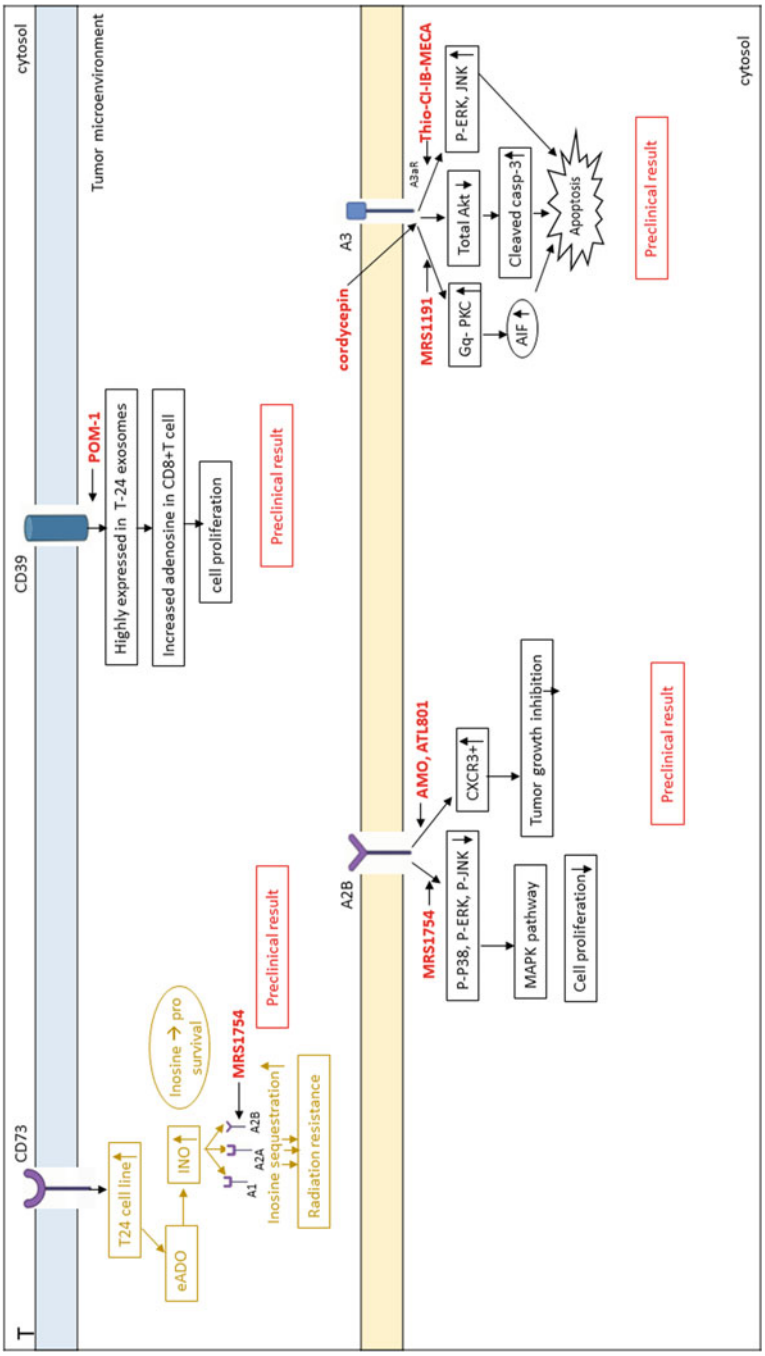


Fig. 19.4 Schematic representation of adenosine receptors, their antagonists and signalling counterparts in urinary bladder carcinoma

A2b receptors may become a potential diagnostic marker as well as a breakthrough drug target for prostate cancer (Mousavi et al. 2015). On the other hand, adenosine A3R activation suppressed prostate cancer metastasis by inhibiting NADPH oxidase activity. A3AR agonist, IB-MECA has been used to inhibit cancer cell growth and metastasis. Furthermore, A3AR mediated regulation of PCa cell proliferation and invasion also involves ERK/MAPK pathway (Jajoo et al. 2009). Both NADPH oxidase suppression and ERK/MAPK pathway activation revolve around the balance between adenylyl cyclase-PKA and ROS generation inside the tumour microenvironment. Vecchio et al. showed that in aggressive PCa cell line, ligand independent constitutive activation of A2Bar contributes significantly in prostate cancer cell proliferation and progression (Vecchio et al. 2016). Constitutive production of cAMP due to A2BAR expression induces the adenylyl cyclase mediated activation of MAPK pathway which may be the reason for prostate cancer aggressiveness. Conversely, as seen in other solid cancers, extracellular adenosine plays crucial role in tumour growth and proliferation. The balance between adenosine converting enzymes CD39 and CD73, and adenosine receptors inside the cellular microenvironment decides how tumour cells will behave (Fig. 19.5).

19.3.2 Adenosine Signalling as an Immunotherapy Regimen in Genitourinary Cancer

The ongoing battery of clinical trials to test the efficacy and safety of different experimental immunotherapeutic drugs has immensely benefited patients as well as helped clinicians with multiple treatment options to choose from (Galsky 2018). Receptors and enzymes involved in adenosine signalling pathway have shown promising results in different in vivo and in vitro experimental models of genitourinary tumour (Novío and Freire-Garabal 2017). Some of these agents are tried along with other immunotherapeutic agents like PD-1/PD-L1 inhibitors or CTL-4 inhibitor (Cattrini et al. 2016).

19.3.3 Adenosine Targeting Therapy in Combination with Immunotherapeutic Agents

Lawrence Fong et al. (Fong et al. 2020) recently published initial results of their phase I dose-escalation study of A2AR receptor antagonist ciforadenant (previously known as CPI-444) in combination with PD-L1 inhibitor atezolizumab in patients with advanced refractory cancers. Early observational evidence indicated strong antitumor activity in patients with treatment refractory RCC. The trial was further expanded to phase Ib to gain more experience with monotherapy and combination therapy in this disease. Out of 68 RCC patients enrolled for the study, 33 received ciforadenant monotherapy while the rest 35 patients received the combination of ciforadenant and atezolizumab. All patients enrolled in the study had progressive disease at the time of study entry and had failed multiple previous therapies

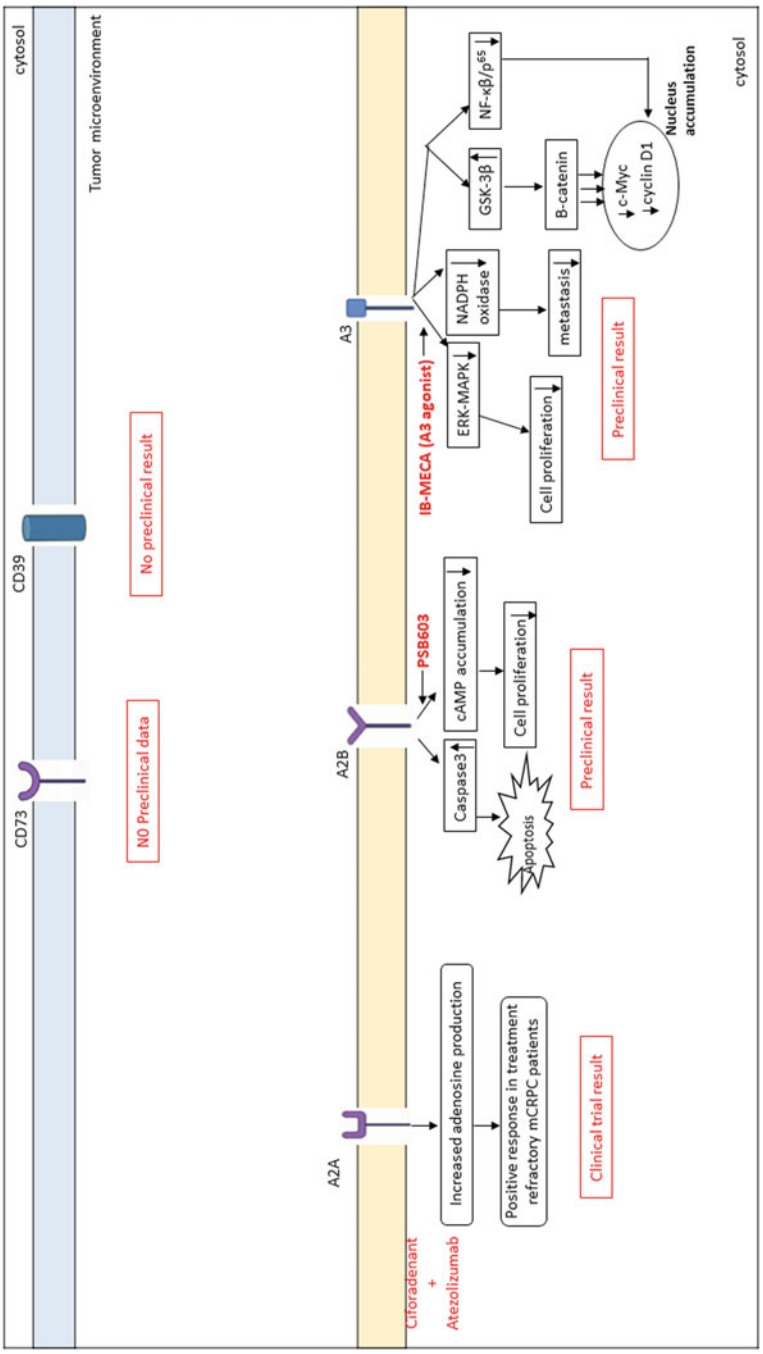


Fig. 19.5 Schematic representation of adenosine receptors, their antagonists and signalling counterparts in prostate cancer (PCa)

including TKIs and anti-PD-1 antibodies. In the ciforadenant monotherapy group, partial response (following RECIST criteria) was achieved in 1 of 33 patients (3%). On the other hand, in the combination treatment group, 4 of 35 (11%) patients achieved personal response. In addition to the above observations, 24% (15 of 63 evaluable) of patients experienced tumour regression but did not meet the RECIST criteria for a partial response. The median progression-free survival was 4.1 months and 5.8 months for ciforadenant monotherapy and combination treatment, respectively. For the combination group, the estimated overall survival (OS) exceeded 90% at 25 months while for the ciforadenant monotherapy group, it was 69% at 16 months. Upon further analysis, it was observed that efficacy of ciforadenant therapy is associated with intratumor $CD8^+$ T-cell infiltration. The adenosine induced genes were denoted as $AdenoSig^{hi}$ and comprised of IL1β, PTGS2, and CXCL1, 2, 3, 5, 6, 8 genes. Interestingly, most of the RCC patients (72%) enrolled in this study were treatment refractory with a median 3 prior treatment. Therefore, the authors concluded that the effectiveness of ciforadenant and other adenosine pathway antagonists can be further extended when used in earlier lines of therapy because of the less exposure of immune system to prior immunosuppressive regimens.

In the same line as RCC, the same group recently *from corvus pharmaceuticals have* recently presented their updated clinical trial data in the recently concluded American Society of Clinical Oncology, 2020 Genitourinary Cancers Symposium (ASCO-GU) in San Francisco (Harshman et al. 2020). The study was a Phase 1b/2 clinical trial of ciforadenant in patients with metastatic castration resistant prostate cancer (mCRPC) as a monotherapy as well as in combination with PD-L1 inhibitor atezolizumab. The clinical details included data from 35 patients with advanced mCRPC, including 11 that received ciforadenant as a monotherapy (100 mg twice daily) and 24 that received ciforadenant (100 mg twice daily) in combination with atezolizumab (840 mg delivered intravenously every 2 weeks). After a 3.2+ months of median follow up, one patient with partial response (PR) was observed as per the RECIST criteria with a prostate-specific antigen (PSA) level dropped from 98 to less than 1. Additionally, tumour regression was seen in 10 patients, but these did not meet the RECIST criteria for PR. 8 pts had stable disease (SD) for a clinical benefit rate of 8/14 (57%). Median duration of disease control was 24 weeks. Gene expression profiling of tumour biopsies revealed a significant correlation of tumour CD73 expression with the adenosine signature ($p = 0.02$). This result indicates that adenosine induced immunosuppressive genes are crucial for antitumor immunity inside the tumour microenvironment.

19.4 Adenosinergic Pathway Targeting Drugs Currently in Clinical Trial for Genitourinary Cancer

Adenosine signalling pathway is considered as the new age immunotherapeutic targets in solid cancer owing to the excellent preclinical results both in vitro and in vivo. Since last 2 years, number of clinical trials with adenosine signalling

targeting agents have increased considerably in different solid cancer types. A2aR, CD73 and CD39 targeting agents are undergoing clinical trials in most genitourinary malignancies. Couple of these trials recently published their initial results on mCRPC and ccRCC as discussed in earlier section. Pharma giants are feeding the market with different experimental drugs targeting adenosine pathway with either other immunotherapeutic agents or chemotherapeutic drugs. The list of the ongoing clinical trials with targeting genitourinary malignancies are listed below (Table 19.2).

19.5 Summary and Future Direction

Thus, it is clear that adenosine pathways could be a breakthrough immunotherapeutic pathway, especially in genitourinary malignancies based upon preclinical and clinical studies. However, we need to wait for the full-fledged results from ongoing clinical trials. Furthermore, it has been seen that CD73 along with CD39 are important for maintaining adenosine balance in healthy cells (Ghalamfarsa et al. 2019). So, caution needs to be exercised while trying antagonists of these targets. Overall, adenosine pathway antagonists can become a new addition in the current armamentarium of immunotherapeutic agents against genitourinary cancers, benefitting patients.

Table 19.2 Ongoing clinical trials with experimental agents targeting adenosine signalling pathway in genitourinary cancers

Serial no.	Molecular target	Clinical trial identifier	Pharmaceutical agent	Organisation/ company	Trial type	Targeted cancer	No. of participants
1	A2AR	NCT03207867 (clinicaltrials.gov 2020)	NIR178 + PDR001	Novartis	A Phase 2, multi-center, open label study	Advanced solid malignancy including ccRCC, mCRPC and urothelial cancer and non-Hodgkin lymphoma	376
2	A2AR + CD73	NCT04089553 (An Open-label, Phase II Study of AZD4635 in Patients With Prostate Cancer n.d.)	AZD4635 + oleclumab	AstraZeneca	Open label, phase-2 modular study	Metastatic castration resistant prostate cancer (mCRPC)	90
3	A2AR + CD73 + PD-1	NCT03549000 (A Phase I/Ib Study of NZV930 Alone and in Combination With PDR001 and or NIR178 in Patients With Advanced Malignancies n.d.)	NIR178 + NZV930 + PDR001 (spartalizumab)	Novartis	A Phase I/Ib, open-label, multi-center, study	Advanced solid malignancy including ccRCC, mCRPC	

(continued)

Table 19.2 (continued)

Serial no.	Molecular target	Clinical trial identifier	Pharmaceutical agent	Organisation/ company	Trial type	Targeted cancer	No. of participants
4	CD73 + PD-1	NCT04148937 (A Study of the CD73 Inhibitor LY3475070 Alone or in Combination With Pembrolizumab in Participants With Advanced Cancer n.d.)	LY3475070 + Pembrolizumab	Eli Lilly and Company	A Phase 1 multicenter global first in human study; Randomize, parallel assignment	Advanced solid malignancy including ccRCC, mCRPC	150
5	CD73 + A2aR + PD-1	NCT03454451 (CPI-006 Alone and in Combination With Ciforadenant and With Pembrolizumab for Patients With Advanced Cancers n.d.)	CPI006 + Ciforadenant + pembrolizumab	Corvus Pharmaceuticals, Inc.	A phase 1/1b multicenter study; randomized and sequential assignment	Advanced solid malignancy including ccRCC, mCRPC and urothelial cancer	378
6	CD73 + PD-L1 (MIBC)	NCT03773666 (A Feasibility Study of Durvalumab +/ − Oleclumab as Neoadjuvant Therapy for Muscle-invasive Bladder Cancer (BLASST-2) n.d.)	Oleclumab + durvalumab	Dana-Farber Cancer Institute along with AstraZeneca	Non-randomized, parallelly assigned, feasibility study	Confirmed case of MIBC patients before surgery without chemotherapy	24

7	CD73 + PD-L1	NCT04262375 (A Phase 2 Study of Durvalumab (MEDI4736) and Oleclumab (MEDI9447) in Multi-Cancer Populations With Correlation to Clinical, Molecular and Immunologic Parameters With DNA MethylaTION n.d.)	Oleclumab + durvalumab	University health network, Toronto with AstraZeneca	Single group assignment; phase 2 multicentre study	Patients with previously treated advanced/ metastatic NSCLC or renal cell cancer will be recruited in near equal distribution	55
8	CD39 + PD-1 + chemotherapy	NCT04306900 (TTX-030 in Combination With Immunotherapy and/or Chemotherapy in Subjects With Advanced Cancers n.d.)	TTX-030 + Budigalimab + docetaxel	Trishula Therapeutics, Inc. with AbbVie	Randomized, single group assignment phase 1/1b study	Advanced solid malignancy including ccRCC, mCRPC	152

(continued)

Table 19.2 (continued)

Serial no.	Molecular target	Clinical trial identifier	Pharmaceutical agent	Organisation/ company	Trial type	Targeted cancer	No. of participants
9	CD39 + PD-1 + chemotherapy (MIBC + prostate)	NCT03884556 (TTX-030 Single Agent and in Combination With Immunotherapy or Chemotherapy for Patients With Advanced Cancers n.d.)	TTX-030 + pembrolizumab + gemcitabine + docetaxel + paclitaxel	Trishula Therapeutics, Inc.	Randomized, sequentially assigned, phase 1/1b safety study	Advanced solid malignancy including mCRPC and MIBC	100
10	A2aR + PD-L1 (ccRCC + prostate + urogenital neoplasm)	NCT02655822 (Phase 1/1b Study to Evaluate the Safety and Tolerability of Ciforadenant Alone and in Combination With Atezolizumab in Advanced Cancers n.d.)	Ciforadenant + atezolizumab	Corvus Pharmaceuticals, Inc. with genentech	Randomized, parallelly assigned, phase 1/1b, open-label, multicentred, repeat-dose, dose-selection study	Advanced solid malignancy including ccRCC, mCRPC	336
11	A2aR + CD73 + (PD-L1) (prostate cancer)	NCT02740985 (A Phase 1 Clinical Study of AZD4635 in Patients With Advanced Solid Malignancies n.d.)	AZD4635 + durvalumab + oleclumab + abiraterone acetate + enzalutamide	AstraZeneca	Non-randomized, parallelly assigned, phase 1, open-label, multicentred study to assess safety, tolerability, pharmacokinetics	Advanced solid malignancies including mCRPC	313

References

A Feasibility Study of Durvalumab +/− Oleclumab as Neoadjuvant Therapy for Muscle-invasive Bladder Cancer (BLASST-2) (n.d.). https://ClinicalTrials.gov/show/NCT03773666

A Phase 1 Clinical Study of AZD4635 in Patients With Advanced Solid Malignancies (n.d.). https://ClinicalTrials.gov/show/NCT02740985

A Phase 2 Study of Durvalumab (MEDI4736) and Oleclumab (MEDI9447) in Multi-Cancer Populations With Correlation to Clinical, Molecular and Immunologic Parameters With DNA MethylaTION (n.d.). https://ClinicalTrials.gov/show/NCT04262375

A Phase I/Ib Study of NZV930 Alone and in Combination With PDR001 and or NIR178 in Patients With Advanced Malignancies (n.d.). https://ClinicalTrials.gov/show/NCT03549000

A Study of the CD73 Inhibitor LY3475070 Alone or in Combination With Pembrolizumab in Participants With Advanced Cancer (n.d.). https://ClinicalTrials.gov/show/NCT04148937

Allard B, Longhi MS, Robson SC, Stagg J (2017) The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immunol Rev 276(1):121–144

Allard B, Allard D, Buisseret L, Stagg J (2020a) The adenosine pathway in immuno-oncology. Nat Rev Clin Oncol 17(10):611–629

Allard D, Allard B, Stagg J (2020b) On the mechanism of anti-CD39 immune checkpoint therapy. J Immunother Cancer 8(1):e000186

An Open-label, Phase II Study of AZD4635 in Patients With Prostate Cancer (n.d.). https://ClinicalTrials.gov/show/NCT04089553

Antonioli L, Fornai M, Blandizzi C, Pacher P, Haskó G (2019) Adenosine signaling and the immune system: when a lot could be too much. Immunol Lett 205:9–15

Arab S, Hadjati J (2019) Adenosine blockage in tumor microenvironment and improvement of cancer immunotherapy. Immune Netw 19(4):e23

Blay J, White TD, Hoskin DW (1997) The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. Cancer Res 57(13):2602–2605

Boison D, Yegutkin GG (2019) Adenosine metabolism: emerging concepts for cancer therapy. Cancer Cell 36(6):582–596

Boyle J (2005) Lehninger principles of biochemistry (4th ed.): Nelson, D., and Cox, M. Biochem Mol Biol Educ 33(1):74–75

Cairns P (2010) Renal cell carcinoma. Cancer Biomark 9(1–6):461–473

Canale FP, Ramello MC, Núñez N, Furlan CLA, Bossio SN, Serrán MG et al (2018) CD39 expression defines cell exhaustion in tumor-infiltrating $CD8^+$ T cells. Cancer Res 78(1):115–128

Cao HL, Liu ZJ, Chang Z (2017) Cordycepin induces apoptosis in human bladder cancer cells via activation of A3 adenosine receptors. Tumour Biol 39(7):1010428317706915

Cattrini C, Dellepiane C, Cavo A, Buzzatti G, Tolomeo F, Messina C et al (2016) Immunotherapy for genitourinary cancer: state of the art and new perspectives. Anti-Cancer Drugs 27(7):585–599

Cekic C, Sag D, Li Y, Theodorescu D, Strieter RM, Linden J (2012) Adenosine A2B receptor blockade slows growth of bladder and breast tumors. J Immunol (Baltimore, Md: 1950) 188(1):198–205

Challier J, Bruniquel D, Sewell AK, Laugel B (2013) Adenosine and cAMP signalling skew human dendritic cell differentiation towards a tolerogenic phenotype with defective CD8(+) T-cell priming capacity. Immunology 138(4):402–410

Chen J-F, Eltzschig HK, Fredholm BB (2013) Adenosine receptors as drug targets—what are the challenges? Nat Rev Drug Discov 12(4):265–286

clinicaltrials.gov (2020). https://clinicaltrials.gov/ct2/show/NCT04342897?term=Eli+Lilly & cond=LY3127804 & draw=2 & rank=1

Cohen HB, Briggs KT, Marino JP, Ravid K, Robson SC, Mosser DM (2013) TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses. Blood 122(11):1935–1945

CPI-006 Alone and in Combination With Ciforadenant and With Pembrolizumab for Patients With Advanced Cancers (n.d.). https://ClinicalTrials.gov/show/NCT03454451

Dellê H, Ferreira JM, Matheus LH, Leite KR, Murta CB, de Almeida Claro JF, Camacho CP, Pontes-Júnior J (2020) Expression balance of ectoenzymes CD39 and CD73 predicts invasiveness in human bladder cancer. https://doi.org/10.21203/rs.3.rs-39791/v1

Dietrich F, Figueiró F, Filippi-Chiela EC, Cappellari AR, Rockenbach L, Tremblay A et al (2018) Ecto-5′-nucleotidase/CD73 contributes to the radiosensitivity of T24 human bladder cancer cell line. J Cancer Res Clin Oncol 144(3):469–482

Donaldson SH, Picher M, Boucher RC (2002) Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. Am J Respir Cell Mol Biol 26(2):209–215

Duhen T, Duhen R, Montler R, Moses J, Moudgil T, de Miranda NF et al (2018) Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat Commun 9(1):2724

Feng L-L, Cai Y-Q, Zhu M-C, Xing L-J, Wang X (2020) The yin and yang functions of extracellular ATP and adenosine in tumor immunity. Cancer Cell Int 20:110

Ferretti E, Horenstein A, Canzonetta C, Costa F, Morandi F (2018) Canonical and non-canonical adenosinergic pathways. Immunol Lett 205:25–30

Fong L, Hotson A, Powderly JD, Sznol M, Heist RS, Choueiri TK et al (2020) Adenosine 2A receptor blockade as an immunotherapy for treatment-refractory renal cell cancer. Cancer Discov 10(1):40–53

Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ 14(7):1315–1323

Fredholm BB, Ijzerman AP, Jacobson KA, Linden J, Müller CE (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. Pharmacol Rev 63(1):1–34

Galsky MD (2018) Advancing care through genomics and immune checkpoint blockade. Nat Rev Urol 15(2):71–72

Ghalamfarsa G, Kazemi MH, Raoofi Mohseni S, Masjedi A, Hojjat-Farsangi M, Azizi G et al (2019) CD73 as a potential opportunity for cancer immunotherapy. Expert Opin Ther Targets 23(2):127–142

Ghiringhelli F, Bruchard M, Chalmin F, Rébé C (2012) Production of adenosine by ectonucleotidases: a key factor in tumor immunoescape. J Biomed Biotechnol 2012:473712

González del Alba A, Arranz JÁ, Puente J, Méndez-Vidal MJ, Gallardo E, Grande E et al (2017) Recent advances in genitourinary tumors: a review focused on biology and systemic treatment. Crit Rev Oncol Hematol 113:171–190

Harshman LC, Chu M, George S, Hughes BGM, Carthon BC, Fong L et al (2020) Adenosine receptor blockade with ciforadenant +/− atezolizumab in advanced metastatic castration-resistant prostate cancer (mCRPC). J Clin Oncol 38(6_Suppl):129

Haschemi A, Wagner O, Marculescu R, Wegiel B, Robson SC, Gagliani N et al (2007) Cross-regulation of carbon monoxide and the adenosine A2a receptor in macrophages. J Immunol 178(9):5921–5929

Haskó G, Szabó C, Németh ZH, Kvetan V, Pastores SM, Vizi ES (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J Immunol (Baltimore, Md: 1950) 157(10):4634–4640

Haskó G, Kuhel DG, Chen J-F, Schwarzschild MA, Deitch EA, Mabley JG et al (2000) Adenosine inhibits IL-12 and TNF-α production via adenosine A2a receptor-dependent and independent mechanisms. FASEB J 14(13):2065–2074

Haskó G, Linden J, Cronstein B, Pacher P (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov 7(9):759–770

Horenstein AL, Chillemi A, Zaccarello G, Bruzzone S, Quarona V, Zito A et al (2013) A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. Oncoimmunology 2(9):e26246

Jadidi-Niaragh F (2019) Potential of CD73 as a target for cancer immunotherapy. Immunotherapy 11(16):1353–1355

Jajoo S, Mukherjea D, Watabe K, Ramkumar V (2009) Adenosine A(3) receptor suppresses prostate cancer metastasis by inhibiting NADPH oxidase activity. Neoplasia (New York, NY) 11(11):1132–1145

Kaczmarek E, Koziak K, Sévigny J, Siegel JB, Anrather J, Beaudoin AR et al (1996) Identification and characterization of CD39/vascular ATP diphosphohydrolase. J Biol Chem 271(51): 33116–33122

Kanno T, Gotoh A, Fujita Y, Nakano T, Nishizaki T (2012) A(3) adenosine receptor mediates apoptosis in 5637 human bladder cancer cells by G(q) protein/PKC-dependent AIF upregulation. Cell Physiol Biochem 30(5):1159–1168

Kim H, Kang JW, Lee S, Choi WJ, Jeong LS, Yang Y et al (2010) A3 adenosine receptor antagonist, truncated Thio-Cl-IB-MECA, induces apoptosis in T24 human bladder cancer cells. Anticancer Res 30(7):2823–2830

Koivisto MK, Tervahartiala M, Kenessey I, Jalkanen S, Boström PJ, Salmi M (2019) Cell-type-specific CD73 expression is an independent prognostic factor in bladder cancer. Carcinogenesis 40(1):84–92

Leclerc BG, Charlebois R, Chouinard G, Allard B, Pommey S, Saad F et al (2016) CD73 expression is an independent prognostic factor in prostate cancer. Clin Cancer Res 22(1):158–166

Li L, Huang L, Ye H, Song SP, Bajwa A, Lee SJ et al (2012) Dendritic cells tolerized with adenosine A_2AR agonist attenuate acute kidney injury. J Clin Invest 122(11):3931–3942

Merighi S, Gessi S, Borea PA (2018) Adenosine receptors: structure, distribution, and signal transduction. In: Merighi S, Gessi S, Borea PA (eds) The adenosine receptors, vol 34, 1st edn. Humana Press, pp 33–57

Miller JS, Cervenka T, Lund J, Okazaki IJ, Moss J (1999) Purine metabolites suppress proliferation of human NK cells through a lineage-specific purine receptor. J Immunol 162(12):7376–7382

Morello S, Capone M, Sorrentino C, Giannarelli D, Madonna G, Mallardo D et al (2017) Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. J Transl Med 15(1):244

Mousavi S, Panjehpour M, Izadpanahi MH, Aghaei M (2015) Expression of adenosine receptor subclasses in malignant and adjacent normal human prostate tissues. Prostate 75(7):735–747

Müller CE, Jacobson KA (2011) Recent developments in adenosine receptor ligands and their potential as novel drugs. Biochim Biophys Acta 1808(5):1290–1308

Nagaya H, Gotoh A, Kanno T, Nishizaki T (2013) A3 adenosine receptor mediates apoptosis in in vitro RCC4-VHL human renal cancer cells by up-regulating AMID expression. J Urol 189(1): 321–328

Najar HM, Ruhl S, Bru-Capdeville AC, Peters JH (1990) Adenosine and its derivatives control human monocyte differentiation into highly accessory cells versus macrophages. J Leukoc Biol 47(5):429–439

Novío SN-IMJ, Freire-Garabal M (2017) Adenosine signaling pathways as potential therapeutic targets in prostate cancer disease. In: Farooqi AIM (ed) Molecular oncology: underlying mechanisms and translational advancements. Springer, Cham

Novitskiy SV, Ryzhov S, Zaynagetdinov R, Goldstein AE, Huang Y, Tikhomirov OY et al (2008) Adenosine receptors in regulation of dendritic cell differentiation and function. Blood 112(5): 1822–1831

Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MKK et al (2006) A2A adenosine receptor protects tumors from antitumor T cells. Proc Natl Acad Sci U S A 103(35): 13132–13137

Pattanaik S, Dey S, Jaiswal N, Rohilla R, Singh S, Mandal A et al (2019) Efficacy and safety of programmed cell death-1/programmed cell death ligand-1 inhibitors in advanced urothelial malignancy: a systematic review and meta-analysis. Indian J Urol 35(2):101–115

Phase 1/1b Study to Evaluate the Safety and Tolerability of Ciforadenant Alone and in Combination With Atezolizumab in Advanced Cancers (n.d.). https://ClinicalTrials.gov/show/NCT02655822

Phelps PT, Anthes JC, Correll CC (2006) Characterization of adenosine receptors in the human bladder carcinoma T24 cell line. Eur J Pharmacol 536(1–2):28–37

Priebe T, Platsoucas CD, Nelson JA (1990) Adenosine receptors and modulation of natural killer cell activity by purine nucleosides. Cancer Res 50(14):4328–4331

Qi Y, Xia Y, Lin Z, Qu Y, Qi Y, Chen Y et al (2020) Tumor-infiltrating CD39(+)CD8(+) T cells determine poor prognosis and immune evasion in clear cell renal cell carcinoma patients. Cancer Immunol Immunother 69(8):1565–1576

Raskovalova T, Lokshin A, Huang X, Jackson EK, Gorelik E (2006) Adenosine-mediated inhibition of cytotoxic activity and cytokine production by IL-2/NKp46-activated NK cells: involvement of protein kinase A isozyme I (PKA I). Immunol Res 36(1–3):91–99

Sek K, Mølck C, Stewart GD, Kats L, Darcy PK, Beavis PA (2018) Targeting adenosine receptor signaling in cancer immunotherapy. Int J Mol Sci 19(12):3837

Siegel RL, Miller KD, Jemal A (2019) Cancer statistics, 2019. CA Cancer J Clin 69(1):7–34

Simoni Y, Becht E, Fehlings M, Loh CY, Koo S-L, Teng KWW et al (2018) Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. Nature 557(7706): 575–579

Song L, Ye W, Cui Y, Lu J, Zhang Y, Ding N et al (2017) Ecto-5′-nucleotidase (CD73) is a biomarker for clear cell renal carcinoma stem-like cells. Oncotarget 8(19):31977–31992

Stella J, Bavaresco L, Braganhol E, Rockenbach L, Farias PF, Wink MR et al (2010) Differential ectonucleotidase expression in human bladder cancer cell lines. Urol Oncol 28(3):260–267

Street SE, Kramer NJ, Walsh PL, Taylor-Blake B, Yadav MC, King IF et al (2013) Tissue-nonspecific alkaline phosphatase acts redundantly with PAP and NT5E to generate adenosine in the dorsal spinal cord. J Neurosci 33(27):11314–11322

Szabó C, Scott GS, Virág L, Egnaczyk G, Salzman AL, Shanley TP et al (1998) Suppression of macrophage inflammatory protein (MIP)-1alpha production and collagen-induced arthritis by adenosine receptor agonists. Br J Pharmacol 125(2):379–387

TTX-030 in Combination With Immunotherapy and/or Chemotherapy in Subjects With Advanced Cancers (n.d.). https://ClinicalTrials.gov/show/NCT04306900

TTX-030 Single Agent and in Combination With Immunotherapy or Chemotherapy for Patients With Advanced Cancers (n.d.). https://ClinicalTrials.gov/show/NCT03884556

Vaupel P, Mayer A (2016) Hypoxia-driven adenosine accumulation: a crucial microenvironmental factor promoting tumor progression. Adv Exp Med Biol 876:177–183

Vecchio EA, Tan CY, Gregory KJ, Christopoulos A, White PJ, May LT (2016) Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation. J Pharmacol Exp Ther 357(1):36–44

Vigano S, Alatzoglou D, Irving M, Ménétrier-Caux C, Caux C, Romero P et al (2019) Targeting adenosine in cancer immunotherapy to enhance T-cell function. Front Immunol 10:925

Wettstein MS, Buser L, Hermanns T, Roudnicky F, Eberli D, Baumeister P et al (2015) CD73 predicts favorable prognosis in patients with nonmuscle-invasive urothelial bladder cancer. Dis Markers 2015:785461

Whiteside TL (2008) The tumor microenvironment and its role in promoting tumor growth. Oncogene 27(45):5904–5912

Wolberg G, Zimmerman T, Hiemstra K, Winston M, Chu L (1975) Adenosine inhibition of lymphocyte-mediated cytolysis: possible role of cyclic adenosine monophosphate. Science 187(4180):957–959

Yang M, Ma C, Liu S, Shao Q, Gao W, Song B et al (2010) HIF-dependent induction of adenosine receptor A2b skews human dendritic cells to a Th2-stimulating phenotype under hypoxia. Immunol Cell Biol 88(2):165–171

Yang Q, Du J, Zu L (2013) Overexpression of CD73 in prostate cancer is associated with lymph node metastasis. Pathol Oncol Res 19:811–814

Yegutkin GG, Henttinen T, Samburski SS, Spychala J, Jalkanen S (2002) The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. Biochem J 367(1):121–128

Yi Y, Zhou Y, Chu X, Zheng X, Fei D, Lei J et al (2020) Blockade of adenosine A2b receptor reduces tumor growth and migration in renal cell carcinoma. J Cancer 11(2):421–431

Young A, Ngiow SF, Gao Y, Patch AM, Barkauskas DS, Messaoudene M et al (2018) A2AR adenosine signaling suppresses natural killer cell maturation in the tumor microenvironment. Cancer Res 78(4):1003–1016

Yu YI, Wang W, Song L, Hu W, Dong C, Pei H et al (2015) Ecto-5′-nucleotidase expression is associated with the progression of renal cell carcinoma. Oncol Lett 9(6):2485–2494

Zarrabi K, Paroya A, Wu S (2019) Emerging therapeutic agents for genitourinary cancers. J Hematol Oncol 12(1):89

Zhou Y, Tong L, Chu X, Deng F, Tang J, Tang Y et al (2017a) The adenosine A1 receptor antagonist DPCPX inhibits tumor progression via the ERK/JNK pathway in renal cell carcinoma. Cell Physiol Biochem 43(2):733–742

Zhou Y, Chu X, Deng F, Tong L, Tong G, Yi Y et al (2017b) The adenosine A2b receptor promotes tumor progression of bladder urothelial carcinoma by enhancing MAPK signaling pathway. Oncotarget 8(30):48755–48768

Zhou Y, Chu X, Yi Y, Hao Z, Zheng X, Yuxin T et al (2019) MRS1754 inhibits proliferation and migration of bladder urothelial carcinoma by regulating mitogen-activated protein kinase pathway. J Cell Physiol 234(7):11360–11368

Zimmermann H (1992) 5′-Nucleotidase: molecular structure and functional aspects. Biochem J. 285 (Pt 2):345–365

Zumerle S, Calì B, Munari F, Angioni R, Di Virgilio F, Molon B et al (2019) Intercellular calcium signaling induced by ATP potentiates macrophage phagocytosis. Cell Rep 27(1):1–10.e4

Immune Checkpoint Inhibitors in Cancer Therapy: A Ray of Hope

20

Chandan Krushna Das and Shrawan K. Singh

Abstract

Immune checkpoint inhibitors (ICIs) have revolutionized the approach to cancer management in the recent decade. In the physiological state, the dynamic interplay between persistent death receptor 1 and its ligand modulates the immune tolerance to self-antigens and adaptive immune response to neoantigens. The rapid development and regulatory approval of newer immune checkpoint inhibitors targeting CTLA-4 and PD-1/PD-L1 lead to a paradigm shift in primary cancer treatments, with some of them achieving a cure. Despite striking and sustainable responses in most patients, eventually, they develop treatment resistance and cancer progression. The factors for the prediction of clinical efficacy of checkpoint inhibitors are complex and evolving. This chapter features immune checkpoint inhibitors in solid cancer management and prediction of treatment response.

Keywords

Cancer · Immune checkpoint inhibitor · Immune system · Tumor microenvironment · Drug resistance

The key achievement in cancer treatment in the last decade has unequivocally been the introduction of immune-checkpoint inhibitors targeting Cytotoxic

C. K. Das
Department of Clinical Hematology and Medical Oncology, Post Graduate Institute of Medical Sciences and Research, Chandigarh, India

S. K. Singh (✉)
Department of Urology, Post graduate Institute of Medical Education and Research, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_20

T-lymphocyte–associated antigen 4 (CTLA-4) and Persistent Death receptor 1 (PD1) or Persistent Death receptor Ligand 1 (PDL1). Ipilimumab, the antibody targeting CTLA4 was approved in 2011 for metastatic melanoma. This seminal event led to the development of other antibodies targeting PD1/PL1 at an unprecedented speed. A decade later, more than 4000 clinical trials actively evaluating immune checkpoint inhibitors with and without chemotherapy.

Previously, immunotherapy was in the form of a cancer vaccine, and cytokine therapies were toxic and ineffective. There was a passivizing in the developmental research of cancer immune therapy. With the understanding of cancer checkpoints, especially the PD1/PDL1 axis, it heralded a new era of ICI therapy. The paradigm shift in approach to cancer treatment led to the 2018 Nobel Award in medicine to James Allison and TasukuHonjo (The Nobel Prize in Physiology or Medicine 2018).

In addition to cancer chemotherapy, oncological surgery, and radiotherapy, immunotherapy is now considered the fourth pillar of cancer therapy. Immune checkpoint inhibitors (ICIs) have revolutionized the management of various solid/hematologic malignancies and are used as first-line and as subsequent therapies.

Persistent Death receptor 1 PD-1 (Cluster of differentiation CD279), highly expressed on activated lymphocytes, plays a pivotal role in inducing cancer immune-tolerance by binding to its ligands PD-L1 and PD-L2 on cancer cells. PDL1 is a transmembrane protein expressed in antigen-presenting cells and regulated by proinflammatory cytokines like interferon γ (IFN) (Keir et al. 2008). Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) or Cluster of differentiation CD152 is a part of the immunoglobulin superfamily expressed constitutively in T regulatory cells and conventional T cells only after priming. CTLA-4 eliminates seemingly autoreactive T cells at the early stage of activation in lymph nodes (Brunet et al. 1987).

Under physiological conditions, the PD1/PDL1 axis and other co-immune checkpoints modulate the immune system to prevent autoimmunity and allergy. Along with a variety of oncometabolite enzyme indoleamine 2,3-dioxygenase 1 (IDO1), lymphocyte activating three sLAG3, PD-1/PD-L1 mechanism creates an immune inhibitory environment to evade immune surveillance and accelerate cancer growth. Recent translational studies indicate the PD-L1/PD-1 axis in the cancer microenvironment modulates anti-cancer immunity by T regulatory cell recruitment, T cell mechanistic dysfunction, premature apoptosis, interleukin 10 (IL10) mediated resistance to CD8 +T cell-induced killing (Zou and Chen 2008).

So, the PD-1/PDL1 axis represents a legitimate target for newer anti-cancer therapy like immune checkpoint inhibitor.

The kinetics of ICI response involving the recruitment of lymphocyte and, finally, resultant tumor cell killing. The time to response or reduction of tumor volume exhibit a time lag. This time lag account for the so-called banana effect is seen in the ICI clinical trials. This apparent pseudoprogression is due to immune infiltration and may be classified as progression using conventional Response Evaluation Criteria In Solid Tumors (RECIST) criteria. In cancers with higher mutation signature like melanoma, it is possible to continue remission for an extended period despite the stoppage of the drug due to the priming of lymphocytes from the lymph nodes.

Estimating median PFS/Overall survival (OS) based on conventional RECIST 1.1 potentially understates ICI's efficacy in estimating delayed clinical effect and long-term survival (Schnipper and Schilsky 2018). Therefore, there is a need for new response evaluation criteria while using ICIs such as immune RECIST (Seymour et al. 2017). Alternative statistical models, such as progression-free survival 2 (PFS2), milestone analysis, weighted log-rank test, and Weibull distribution, are upcoming to assess delayed effect and prolonged survival of ICI (Chen 2015).

One of ICI's most impressive successes has been long-term remission despite treatment discontinuation, raising substantial hope for a cure for some patients (Gunasekaran et al. 2020). The long-term remission of metastatic cancer was unimaginable before the era of ICI (Gunasekaran et al. 2020).

The striking thing about IC is a new set of immune-mediated toxicities that are drastically different from the traditional cytotoxic chemotherapies. The classic toxicities are due to the activation of host immunity and the resultant myriad of symptoms resembling autoimmune disorder, including pneumonitis, hypophysitis, and dermatitis. On the combination of CTLA and anti-PD1 inhibitor, the toxicity can reach 50% but rarely reach fatality. This unique array of treatment-emergent adverse effects has warranted an urgent and comprehensive evaluation of symptoms and coordination between different specialties (Johnson et al. 2020).

20.1 Immune Checkpoint Inhibitors in Cancer Therapeutics

Over the last decade, immune checkpoint inhibitors have been approved as the first or subsequent therapy in a wide spectrum of solid and hematological malignancies. The Food and Drug Administration (FDA)-approved immune checkpoint inhibitors most commonly used were listed in the table with their indications (Table 20.1).

Ipilimumab is a CTLA4 ab designed to prevent T cell priming in the lymph nodes. This costimulatory blocker is the first ICI to be approved for any solid cancer. The FDA-approved indications for ipilimumab are melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSCLC) (Seidel et al. 2018).

Nivolumab is the IgG4a anti-PD1 antibody. The FDA-approved indications are Hodgkin's lymphoma, squamous cell carcinoma head and neck (SCCHN), NSCLC, urothelial cancer (UC), RCC, and melanoma (Seidel et al. 2018).

Pembrolizumab is a compact, asymmetrical Y-shaped IgG4 antibody against PD1. It exhibits the Fab exchange mechanism and behaves as dynamic bispecific antibodies. The FDA-approved indications are NSCLC, SCCHN, RCC, HCC, UC, and tumor agnostic approval in microsatellite high cancer and tumor mutational burden (TMB) high cancers (Seidel et al. 2018).

Atezolizumab: It is an anti-PDL1 antibody with a modified crystallizable fragment (Fc) domain to prevent a reduction in PDL1 expressing T cell. It has a minimal binding affinity toward PDL2. In preclinical studies, Atezolizumab impacts the local cytokine milieu by altering interferon-gamma (IFNγ), Interleukin 6 (IL6), and Interleukin 18 (IL 18.) Food and Drug Administration (FDA) approved Atezolizumab in malignant melanoma, NSCLC, extended stage small cell carcinoma

Table 20.1 FDA-approved immunocheckpoint inhibitors in different malignancies

Cancer	Immune checkpoint inhibitor	Line of therapy	PDL1 cut-off for analysis	Trial	Results
NSCLC stage IV	Nivolumab + ipilimumab	Front line EGFR-ALK-	>1%	Checkmate 227 (Hellmann et al. 2019)	*Nivolumab-Ipililmumab: OS: 17.1 months (95% [CI], 15.0–20.1) Chemotherapy 14.9 months (95% CI, 12.7–16.7) (P = 0.007)*
NSCLC stage IV	Nivolumab	Progression on or after platinum-based therapy	≥1%, ≥5%, and ≥10%	Checkmate 057/Checkmate 017 (Gettinger et al. 2019)	*Nivolumab 5 year OS 13.4% Docetaxel 5 year OS 2.6%*
NSCLC stage IV	Atezolizumab + carboplatin + paclitaxel + bevacizumab	EGFR-ALK-metastatic non-squamous NSCLC	TC1/2/3 IC 1/2/3	IMpower150 (Socinski et al. 2018)	*Atezolizumab-Bevacizumab-Chemotherapy 19.2 m Bevacizumab-Chemotherapy Chemotherapy 14.7 m HR 0.78; 95% CI, 0.64–0.96; P = 0.02*
NSCLC stage IV	Atezolizumab	PDL1 Positive metastatic Non-squamous NSCLC	TC ≥50% or IC ≥10%	IMpower110 (Herbst et al. 2020a)	*Atezolizumab OS 20.2, (95%CI 16.5-NE) Chemotherapy OS13.1 m (95% CI 7.4–16.5) (HR, 0.595; CI 0.4–0.89 P = 0.0106)*
NSCLC stage IV	Atezolizumab	Progressed on platinum-based therapy for stage IIIB or IV NSCLC	≥1%	OAK (Fehrenbacher et al. 2018)	*Atezolizumab OS 13.8 M (95% CI 11.8–15.7) Docetaxel 9.6 m (95% CI 8.6–11.2) HR 0.74 95% CI 0.63–0.87 P = 0.0004*

NSCLC stage IV	Pembrolizumab	Stage III NSCLC unfit for surgery/ CTRT or ALK-EGFR-metastatic disease	TPS >50%	KEYNOTE-024 (Reck et al. 2019)	*Pembrolizumab OS: 30.0 months (95% CI, 18.3—not reached) Placebo 14.2 m (95% CI, 9.8–19.0 months) HR 0.63; 95% CI, 0.47–0.86)*
NSCLC stage IV	Pembrolizumab	Progression on or after platinum-containing chemotherapy	TPS ≥1%	KEYNOTE-010 (Herbst et al. 2020b)	*Pembrolizumab 3 year OS 22.9% Docetaxel 3 year OS 11% HR, 0.69; 95% CI, 0.60–0.80; $P < 0.00001$*
NSCLC non-squamous stage IV	Pembrolizumab + pemetrexed + platinum	Metastatic non-squamous EGFR-ALK-NSCLC	≥1% and ≥50%	KEYNOTE 189 (Gadgeel et al. 2020)	*Pembrolizumab + chemotherapy OS 22 m (95%CI 19.5–25.2) Placebo + chemotherapy OS 10.7 m (95%CI 8.7–13.6) HR 0.56; 95% CI, 0.45–0.70 $p < 0.001$*
NSCLC squamous stage IV	Pembrolizumab + carboplatin + paclitaxel/ NabPaclitaxel	Metastatic squamous cell histology NSCLC frontline	≥1%	KEYNOTE 407 (Paz-Ares et al. 2018)	*Pembrolizumab + chemotherapy OS 15.9 m (95% confidence interval [CI], 13.2 to not reached Placebo + chemotherapy 11.3 months (95% CI, 9.5–14.8) (HR for death, 0.64; 95% CI, 0.49–0.85; $P < 0.001$)*
NSCLC stage III	Durvalumab	Post CTRT Maintainance	NA	PACIFIC 1 (Gray et al. 2019)	*Durvalumab OS not reached (95% CI, 34.7-NR) Placebo 28.7 m (95% CI, 22.9-NR) HR = 0.68; 95% CI, 0.53–0.87 ($P = 0.0025$)*

(continued)

Table 20.1 (continued)

Cancer	Immune checkpoint inhibitor	Line of therapy	PDL1 cut-off for analysis	Trial	Results
SCLC-ES	Nivolumab	Post platinum progression third line	NA	CHECKMATE 032 (Ready et al. 2019)	*ORR 12%* *OS @18 month 20%*
SCLC-ES	Durvalumab + carboplatin + etoposide	Extensive stage SCLC	NA	CASPIAN (Paz-Ares et al. 2019)	*Durvalumab + chemotherapy 13 m (95% CI, 11.5–14.8)* *Placebo + chemotherapy 10.3 m (95% CI, 9.3–11.2)* *(HR = 0.73; 95% CI, 0.59–0.91; P = 0.0047)*
SCLC-ES	Atezolizumab + carboplatin + etoposide	Extensive stage SCLC	NA	IMpower133 (Horn et al. 2018)	*Atezolizumab + chemotherapy 12.3 m 95%CI 10.8–15.9* *Placebo + chemotherapy 10.3 m 955CI 9.3–11.3* *HR = 0.70; 95% CI, 0.54, 0.91; P = 0.0069)*
SCLC-ES	Pembrolizumab	Disease progression on or after platinum-based and one other line of therapy chemotherapy third line	NA	KEYNOTE-158 AND KEYNOTE-028 (Chung et al. 2020)	*ORR 19% (95%CI 11–29)* *DoR not reached (41–35.8+)*
Melanoma metastatic	Nivolumab +/− ipilimumab	Unrescectable or metastatic front line	5%	CHECKMATE 067 (Larkin et al. 2019)	*Nivolumab plus ipilimumab OS: not reached > 60 months (95% CI: 38.2, NR)* *Nivolumab 36.9 months (95% CI: 28.3, NR) Ipilimumab 19.9 months (95% CI: 16.9, 24.6)*

Melanoma metastatic	Pembrolizumab	Unresectable or metastatic melanoma front line	≥1%	KEYNOTE-006 (Robert et al. 2019)	*Pembrolizumab OS 32·7 m (95% CI 24·5–41·6)* *Ipilimumab 15·9 m (13·3–22·0)* *HR 0·73, 95% CI 0·61–0·88, p = 0·00049*
Melanoma stage III	Pembrolizumab	Node positive melanoma post surgery	≥1%	KEYNOTE-054 (Eggermont et al. 2020)	*Pembrolizumab 3 year RFS 64%* *Placebo 3 year RFS 44%* *HR 0.56 95% CI 0.47–0.68*
Melanoma metastatic	Atezolizumab + cobimetinib + vemurafenib	BRAF V600E mutant unresectable stage IIIc–IV melanoma frontline	IC ≥1/ 2/3	IMSPIRE150 (Gutzmer et al. 2020)	Atezolizumab: PFS: 15.1 m (95% CI: 11.4, 18.4) Placebo: 10.6 m (95% CI: 9.3, 12.7) (HR 0.78; 95% CI: 0.63, 0.97; $p = 0.0249$)
Melanoma stage IIIB, IIIC, or IV	Nivolumab	Adjuvant post-surgery	5%	CHECKMATE 238 (Weber et al. 2017)	*Nivolumab 3-year RFS rates: 58% Ipilimumab RFS 3 year: 45%* *HR = 0.68; 95% CI = 0.56–0.82, P < 0.0001*
HodgkinsLymphoma	Nivolumab	Post auto HSCT relapse	NA	CHECKMATE 205 (Armand et al. 2018)	*ORR 69% (95% CI, 63–75%)* *DoR 16.6 m (95% CI, 13.2–20.3 m)* *PFS 14.7 m (95% CI, 11.3–18.5 m)*
Classic Hodgkins lymphoma	Pembrolizumab	Post auto HSCT relapse	NA	Keynote-087 (Zinzani et al. 2019)	*ORR 71.0% (95% CI, 64.3–77.0)* *PFS 13.6 m (95% CI, 11.1–16.7)* *3 year OS 86.4%*
ccRCC	Pembrolizumab + axitinib	Untreated clear cell carcinoma	>1%	KEYNOTE-426 (Rini et al. 2019)	*89.9% in the pembrolizumab–axitinib 1 year OS 89.9%* *Sunitinib 1 year OS 78.3%* *HR = 0.53; 95% CI, 0.38–0.74; P < 0.0001*

(continued)

Table 20.1 (continued)

Cancer	Immune checkpoint inhibitor	Line of therapy	PDL1 cut-off for analysis	Trial	Results
ccRCC	Nivolumab + ipilimumab	Intermediate/poor risk IMDC	>1%	CHECKMATE 214 (Motzer et al. 2019)	*Nivolumab-Ipilimumab OS not reached [95% CI 35·6-not estimable]* *Sunitinib 26·6 months [22·1–33·4]* *HR 0·66 [95%CI 0·54–0·80], p < 0·0001,*
ccRCC	Avelumab + axitinib	Untreated clear cell carcinoma	≥1%	JAVELIN Renal 101 Trial (Choueiri et al. 2020)	*Avelumab + Axitinib 13.3 (95% CI 11.1–15.3)* *Sunitinib 8.0 months (95% CI 6.7–9.8)* *HR 0.69 (95% CI 0.574–0.825); P < 0.0001*
MetastaticccRCC	Nivolumab	Post one or two regimens of antiangiogenic therapy	≥1% and ≥5%	CHECKMATE 025 (Motzer et al. 2020)	*Nivolumab 5-year OS 25.8 month (22.2–29.8)* *Everolimus 5-year OS 19.7 (17.6–22.1)* *HR 0.73 (0.62–0.85)*
SCCHN	Nivolumab	Recurrent/metastatic platinum refractory SCCHN	>1%	CHECKMATE 141 (Ferris et al. 2018)	*Nivolumab 7.7 m (5.7–8.8)* *Cetuximab/docetaxel/MTX 5.1 M (4–6.2)* *HR 0.68 (95% CI 0.54–0.86).*
SCCHN	Pembrolizumab	Recurrent/metastatic platinum refractory SCCHN Frontline	CPS ≥1	KEYNOTE-048 (Burtness et al. 2019)	*Pembrolizumab OS 12.3 m (95% CI, 10.8–14.9)* *Cetuximab + chemotherapy 10.3 m (95% CI, 9.0–11.5)* *HR 0.78 95% CI 0.64–0.96* *P = 0.171*

SCCHN	Pembrolizumab + cisplatin + 5FU	Recurrent/metastatic platinum refractory SCCHN Frontline	CPS ≥1	KEYNOTE-048 (Burtness et al. 2019)	*Pembrolizumab + chemotherapy 13.0 m (95% CI, 10.9–14.7) Cetuximab + Chemotheraoy 10.7 m (95% CI, 9.3–11.7) HR 0·77 [95% CI 0·63–0·93], p = 0·0034)*
UC Metastatic	Nivolumab	Metastatic/locally advanced platinum resistant urothelial carcinoma	NA	CHECKMATE 275 (Ohyama et al. 2019)	*OS: 8.6 m (95% CI 6.1–11.3) PFS: 1.9 m (95% [CI] 1.9–2.3) ORR: 20.4%*
UC Metastatic	Pembrolizumab	Platinum ineligible metastatic UC	CPS ≥10	KEYNOTE-052 (Vuky et al. 2020)	*OS 18.5 m (95% CI, 12.2 = 28.5 m) ORR 47.3% (95% CI, 37.7 = 57)*
UC Metastatic	Pembrolizumab	Progression post-platinum-based chemotherapy	NA	KEYNOTE-045 (Fradet et al. 2019)	*Pembrolizumab median OS 10.1 m (8–12.3) Chemotherapy OS 7.3 m (6.1–8.1) HR 0.7 95% CI 0.57–0.85 P = 0.00015*
UC Metastatic	Atezolizumab	Platinum ineligible metastatic UC	>5%	IMvigor 210 (Rosenberg et al. 2016)	*ORR 23.5% (95%CI 16.2–32.2)*
UC Metastatic	Atezolizumab	Progression on or after platinum-based therapy second line	≥5%	IMvigor210 (Rosenberg et al. 2016)	*ORR 14.8% 95%CI 11.2–19.3 DoR 27.7 m (2.1–33.4+)*
UC Metastatic	Durvalumab	Progression on platinum-based chemotherapy	≥25% TC/IC	Powles et al. (Powles et al. 2017)	*(ORR) 17% (95% CI, 11.9–23.3%)*
UC Metastatic	Avelumab	Post platinum progression	NA	JAVELIN Solid Tumor (Patel et al. 2018)	*ORR 16.1% (95%CI 10.8–22.8)*

(continued)

Table 20.1 (continued)

Cancer	Immune checkpoint inhibitor	Line of therapy	PDL1 cut-off for analysis	Trial	Results
UC Metastatic	Avelumab	Maintenance avelumab post-platinum-based therapy first line		JAVELIN bladder 100 (Meeting Library 2020)	*Avelumab OS 21.4 months* *BSC OS 14.3 months,* *HR 0.69; 95% CI 0.56–0.86;* *p = 0.0005*
High risk NMIBC	Pembrolizumab	*BCG refractory with CIS with or without papillary tumors ineligible or have elected not to undergo cystectomy*	NA	KEYNOTE-057 (Balar et al. 2020)	*CR 41% (95% CI 31–51)* *Median DOR: 16.2 m (range: 0.0+ – 30.4+ months)*
Colon cancer	Nivolumab + low-dose ipilimumab	MSI high	NA	CHECKMATE 142 (Lenz et al. 2020)	*OS at 2 years: 79%* *PFS: At 2 years: 74%* *ORR: 60% (95% CI 44.3–74.3)*
Colon cancer	Pembrolizumab	MSI high	NA	KEYNOTE 177 (Andre et al. 2020)	*Pembrolizumab PFS 16 m* *Chemotherapy PFS 8.2 m* *HR = 0.60; 95% CI, 0.45–0.80* *P = 0.0002*
HCC	Nivolumab	HCC progressed on or were intolerant to sorafenib, CPS A	>1%	CHECKMATE 040 (El-Khoueiry et al. 2017)	*OS 16 month* *ORR 14%*
HCC	Nivolumab + ipilimumab	HCC progressed on or were intolerant to sorafenib, CPS A	>1%	CHECKMATE 040 (He et al. 2020)	*Month OS rate was 37%* *ORR was 31%* *DOR of 17 months; 30-*
HCC	Pembrolizumab	Progression on/intolerance to sorafenib Child-Pugh Score A	NA	KEYNOTE-224 (Kudo et al. 2020)	*OS 13.2 months (95% CI 9.7–15.3)* *PFS 4.9 months (95% CI 3.5–6.7)* *ORR 18.3% (95% CI 11.4–27.1)*

HCC	Atezolizumab + bevacizumab	Unresectable HCC	NA	IMbrave150 (Finn et al. 2020)	*Atezolizumab OS NE* *Sorafenib 13.2 (10.4-NE)* *HR 0.58 (95% CI, 0.42–0.79)* *P < 0.001*
TNBC	Atezolizumab + NabPaclitaxel	Metastatic TNBC front line	>1%	IMpassion 130 (Schmid et al. 2020)	*Atezolizumab + NabPaclitaxel OS 25·0 m (95% CI 19·6–30·7)* *Placebo + NabPaclitaxel 18·0 months (13·6–20·1)* *Stratified HR 0·71, [0·54–0·94]*
Advanced squamous callesophagus	Pembrolizumab	The third line relapsed/metastatic squamous cell ca esophagus	CPS ≥10	KEYNOTE-180 (Shah et al. 2019)	*Pembrolizumab OS 10.3 (95% CI, 7.0–13.5)* *Chemotherapy OS 6.7 (95% CI, 4.8–8.6)* *HR 0.64 (95% CI, 0.46–0.90)*
Ca stomach/GEJ	Pembrolizumab	Third line relapsed Ca gastric/GEJ	CPS ≥1	KEYNOTE-059 (Bang et al. 2019)	*ORR 25.8% (95% CI 11.9–44.6)*
MSI-H/dMMR cancers	Pembrolizumab	Relapsed refractory solid cancer progressed post-therapy	NA	KEYNOTE-158 (Marabelle et al. 2020)	*OS 23.5 m (95% CI, 13.5—not reached). PFS 4.1 months (95% CI, 2.4–4.9 m)* *CRC: ORR 36% (95% CI 26–46)* *Non-CRC: ORR 46% (95% 33–59)*
TMB high cancer	Pembrolizumab	TMB > 10 mb/relapsed solid cancer	NA	KEYNOTE-158 (Diaz et al. 2019)	*OS 11.1 m (8.1–16.1)* *PFS 2.1 m (2.1–3.7)* *ORR (95% CI) 28.3% (20.5–37.3)*
Cervical cancer	Pembrolizumab	PDL1 expressing progression after chemotherapy	CPS ≥1	KEYNOTE-158 (Cohort E) (Chung et al. 2019)	*OS 11 m (95% CI, 9.1–14.1)* *PFS 2.1 m (95% CI, 2.0–2.2),* *ORR 14.6% (95% CI, 7.8–24.2%)*
Merkle cell carcinoma	Pembrolizumab	Advanced Merkel cell carcinoma	>1%	KEYNOTE-017 (Nghiem et al. 2019)	*PFS at 6 m 67% (95% CI, 49–86)* *ORR 56% (95% [CI], 35–76)*

(continued)

Table 20.1 (continued)

Cancer	Immune checkpoint inhibitor	Line of therapy	PDL1 cut-off for analysis	Trial	Results
Merkle cell carcinoma	Avelumab	Advanced Merkel cell carcinoma	>1%	JAVELIN Merkel 200 (D'Angelo et al. 2020)	*OS 12.6 m (95% CI 7.5–17.1) ORR 33.0% (95% CI 23.3–43.8%)*
Cutaneous SCC	Pembrolizumab	Recurrent and metastatic SCC not curable by surgery or RT	NA	KEYNOTE-629 (Grob et al. 2019)	*ORR 34% 95% CI 25–44 DoR > 1% 6 month 69% Median DoR not reached (2.7–13.1+)*

SCC squamous cell carcinoma, *PFS* progression-free survival, *OS* overall survival, *DoR* duration of response, *ORR* overall response, *CRC* colorectal cancer, *TMB* tumor mutational burden, *MMR* mismatch repair, *MSI* microsatellite instability, *NA* not available, *GEJ* gastroesophageal junction, *CPS* combined proportion score, *TNBC* triple-negative breast cancer, *HCC* hepatocellular carcinoma, *BCG* bacilli Calmette-Guerin, *NMIBC* non-muscle invasive bladder cancer, *UC* urothelial cancer, *BSC* best supportive care, *TC/IC* tumor-cell/immune cell, *NSCLC* non-small cell lung cancer, *EGFR* epidermal growth factor receptor, *ALK* anaplastic leukemia kinase, *CTRT* concurrent chemotherapy and radiation therapy, *SCLC* ES small cell lung carcinoma extensive-stage, *ccRCC* clear cell renal cell cancer, *SCCHN* squamous cell carcinoma head and neck, *m* and *M* months

lung (SCLC), hepatocellular carcinoma (HCC), triple-negative breast cancer (TNBC), and urothelial cancer (UC) (Akinleye and Rasool 2019).

Durvalumab is a fully human IgG1 monoclonal antibody against PDL1. It was glycoengineered to prevent antibody dependent cell-mediated cytotoxicity and extremely potent even in subnanomolar concentration (Stewart et al. 2015). FDA has approved it for SCLC, UC, and maintenance strategy in Unresectable stage III NSCLC (Akinleye and Rasool 2019).

Avelumab is a human IgG1 monoclonal antibody with a crystallizable fragment (Fc) region, capable of interacting with natural killer cells to induce tumor-directed antibody-dependent cell-mediated cytotoxicity (ADCC). FDA has approved it for renal cell carcinoma (RCC), UC, and Merkle cell carcinoma (Akinleye and Rasool 2019).

20.2 Predictors of Immune Checkpoint Inhibitor Efficacy

Immune checkpoint inhibitors can induce long-term remission despite discontinuation, raising the ultimate hope for a cure. Often, such expectations were met with frustrations. Only a fraction of patients derive such benefit after using ICI therapies. Therefore, the quest for a perfect predictive biomarker has been a daunting task amid the intricate interaction between cancer microenvironment and PD1/PDL1 axis on different tumor types. The search for the ideal biomarker remains far from successful. There are a few established and evolving biomarkers that can be employed to select patients better.

20.2.1 PDL1 Expression

There is a significant degree of disagreement in deciding PDL1 cutoff in predicting ICI efficacy. The probable reason for the variability is due to the type of sample tested, different companion assay methods, and assessment of different PDL1 expressing cells (Table 20.1).

The reasons for such a variable correlation with PDL1 expression and ICI efficacy are due to its spatial and temporal heterogeneity and the tissue-specific immunomodulation (Mansfield et al. 2016). Immunoblotting of exosomal PDL1 and soluble PDL1 estimation may serve as a non-invasive way to monitor ICI drugs' response in the future (Gunasekaran et al. 2020).

20.2.2 Microsatellite Instability

Microsatellite instability leads to fragileness of DNA and leads to hypermutated phenotype of cancer cells resulting from frameshift deletions due to DNA damaging insults. Cancer with microsatellite instability (MSI) high phenotype has a large tumor mutational burden. The rich expression of neuropeptides on the surface

makes it an attractive target of cytotoxic immune effector cells (Le et al. 2017). Despite the subclonal nature of generated mutations on MMR deficient cancer, the sheer volume of neoepitopes on the cancer cells makes them an attractive target of T cells. The other mechanism of ICI activity in MSI high cancer may also involve independent immune pathways (Alexandrov et al. 2013). The tumor microenvironment of microsatellite unstable patients has a high expression of IDO1,LAG3 and PD1-PDL1 axis making it highly antigenic. Based on Keynote 158 trial pembrolizumab got the tissue agnostic approval by USFDA (Marabelle et al. 2020).

20.2.3 Tumor Mutational Burden

Not every patient who received IO drugs derives benefit. The CD8+T cells recognize the neoantigens if only presented with major histocompatibility complex (MHC) by an antigen-presenting cell (APC). Broadly, cancer-associated antigens can be classified into two broad categories: non-mutated self-antigens and non-synonymous somatic mutations derived from antigens (Heemskerk et al. 2013).

Epigenetics and transcriptional reprogramming lead to aberrant expression of non-mutated proteins that are usually restricted. The response to IO drugs is usually blunted owing to central tolerance. The estimation of non-synonymous single nucleotide variants (nsSNVs) in cancer is of paramount importance and constitutes tumor mutational burden. The response of IO to the tumor with high TMB is phenomenal. The previously published studies have yet to identify a clinically relevant cutoff. Friends of Cancer Research TMB Harmonization Project established $\geq$10 mutations/Mb by whole-exome sequencing to be clinically relevant. After the landmark KEYNOTE 158 study, FDA approved Food and Drug Administration recently approved Pembrolizumab for use in relapsed solid cancer with high TMB as defined by a companion diagnostic study. In the relapsed refractory group of cancer with TMB high, pembrolizumab achieved a phenomenal response rate of 50% at 2 years of follow up (Marabelle et al. 2020). The TMB is a dynamic biomarker and constantly changing due to the complex interaction between cancer cells and immune effector cells. Quantification of change in TMB in the initial 4 weeks and estimation by cell-free DNA are the two robust ways to assess TMB accurately and correlate with ICI activity (Havel et al. 2019; Riaz et al. 2017).

Further refinement of TMB in predicting tumor response can be established by determining the real neoantigen burden. The neoantigen burden is the cancer antigens engaged explicitly by the cytotoxic T cells (Rizvi et al. 2015). At present, the neoantigen burden assessment is marred by low specificity, unable to differentiate intracellular or extracellular position, and the dynamic expression (Schumacher and Schreiber 2015). It might be possible to select the patients better for ICI candidates based on neoantigen prediction in the future.

20.2.4 Immune-Desert Phenotype

Cancers like ovarian cancer and hormone receptor-positive breast cancer are immunologically bland and elicit a minimal response to ICI (Nolan et al. 2017). The mechanism of insufficient ICI activity is due to a marked inhibitory tumor microenvironment. Cancer neoangiogenesis contributes to it to a great extent. Immune-desert cancer can be converted into inflamed cancer by adding anti-vascular endothelial growth factor (VEGF), cyclin-dependent kinase 4/6 (CDK4/6), or poly (ADP-ribose) polymerase (PARP) inhibitors to ICI combination. The synergistic combinations are yielding excellent tumor control rates and are being explored in ongoing clinical trials (Ghisoni et al. 2019).

20.3 Conclusions

Finally, after a decade of development, ICI is now established as the mainstay of cancer treatment. Despite the unprecedented rate of cancer trial development and long-term remission, ICI has not yet achieved the cure we all strive for. With immune-checkpoint immunotherapy, a ray of hope for those millions of cancer patients is just opened. In the near future, more accurate and predictive biomarkers may be discovered to maximize ICI therapy.

References

Akinleye A, Rasool Z (2019) Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. J Hematol Oncol 12(1):92

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV et al (2013) Signatures of mutational processes in human cancer. Nature 500(7463):415–421

Andre T, Shiu K-K, Kim TW, Jensen BV, Jensen LH, Punt CJA et al (2020) Pembrolizumab versus chemotherapy for microsatellite instability-high/mismatch repair deficient metastatic colorectal cancer: the phase 3 KEYNOTE-177 Study. J Clin Oncol 38(18_Suppl):LBA4

Armand P, Engert A, Younes A, Fanale M, Santoro A, Zinzani PL et al (2018) Nivolumab for relapsed/refractory classic Hodgkin lymphoma after failure of autologous hematopoietic cell transplantation: extended follow-up of the multicohort single-arm phase II CheckMate 205 trial. J Clin Oncol 36(14):1428–1439

Balar AV et al (2020) Keynote 057: phase II trial of Pembrolizumab (pembro) for patients (pts) with high-risk (HR) non-muscle invasive bladder cancer (NMIBC) unresponsive to bacillus calmette-guérin (BCG). J Clin Oncol [Internet]. [Cited 2020 Sept 8]. https://ascopubs.org/doi/abs/10.1200/JCO.2019.37.7_suppl.350

Bang Y-J, Kang Y-K, Catenacci DV, Muro K, Fuchs CS, Geva R et al (2019) Pembrolizumab alone or in combination with chemotherapy as first-line therapy for patients with advanced gastric or gastroesophageal junction adenocarcinoma: results from the phase II nonrandomized KEYNOTE-059 study. Gastric Cancer 22(4):828–837

Brunet JF, Denizot F, Luciani MF, Roux-Dosseto M, Suzan M, Mattei MG et al (1987) A new member of the immunoglobulin superfamily—CTLA-4. Nature 328(6127):267–270

Burtness B, Harrington KJ, Greil R, Soulières D, Tahara M, de Castro G et al (2019) Pembrolizumab alone or with chemotherapy versus cetuximab with chemotherapy for recurrent

or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-048): a randomised, open-label, phase 3 study. Lancet 394(10212):1915–1928

Chen T-T (2015) Milestone survival: a potential intermediate endpoint for immune checkpoint inhibitors. J Natl Cancer Inst 107(9):djv156

Choueiri TK, Motzer RJ, Rini BI, Haanen J, Campbell MT, Venugopal B et al (2020) Updated efficacy results from the JAVELIN Renal 101 trial: first-line avelumab plus axitinib versus sunitinib in patients with advanced renal cell carcinoma. Ann Oncol 31(8):1030–1039

Chung HC, Ros W, Delord J-P, Perets R, Italiano A, Shapira-Frommer R et al (2019) Efficacy and safety of pembrolizumab in previously treated advanced cervical cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 37(17):1470–1478

Chung HC, Piha-Paul SA, Lopez-Martin J, Schellens JHM, Kao S, Miller WH et al (2020) Pembrolizumab after two or more lines of previous therapy in patients with recurrent or metastatic SCLC: results from the KEYNOTE-028 and KEYNOTE-158 studies. J Thorac Oncol 15(4):618–627

D'Angelo SP, Bhatia S, Brohl AS, Hamid O, Mehnert JM, Terheyden P et al (2020) Avelumab in patients with previously treated metastatic Merkel cell carcinoma: long-term data and biomarker analyses from the single-arm phase 2 JAVELIN Merkel 200 trial. J Immunother Cancer 8(1): e000674

Diaz LA, Le D, Maio M, Ascierto PA, Geva R, Motola-Kuba D et al (2019) Pembrolizumab in microsatellite instability high cancers: updated analysis of the phase II KEYNOTE-164 and KEYNOTE-158 studies. Ann Oncol 30:v475

Eggermont AM, Blank CU, Mandalà M, Long GV, Atkinson V, Dalle S et al (2020) Pembrolizumab versus placebo after complete resection of high-risk stage III melanoma: new recurrence-free survival results from the EORTC 1325-MG/Keynote 054 double-blinded phase III trial at three-year median follow-up. J Clin Oncol 38(15_Suppl):10000

El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C et al (2017) Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet 389(10088):2492–2502

Fehrenbacher L, von Pawel J, Park K, Rittmeyer A, Gandara DR, Ponce Aix S et al (2018) Updated efficacy analysis including secondary population results for OAK: a randomized phase III study of atezolizumab versus docetaxel in patients with previously treated advanced non-small cell lung cancer. J Thorac Oncol 13(8):1156–1170

Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L et al (2018) Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral Oncol 81:45–51

Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y et al (2020) Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N Engl J Med [Internet] 382(20): 1894–1905. [Cited 2020 Sept 9]. https://www.nejm.org/doi/10.1056/NEJMoa1915745

Fradet Y, Bellmunt J, Vaughn DJ, Lee JL, Fong L, Vogelzang NJ et al (2019) Randomized phase III KEYNOTE-045 trial of pembrolizumab versus paclitaxel, docetaxel, or vinflunine in recurrent advanced urothelial cancer: results of >2 years of follow-up. Ann Oncol 30(6):970–976

Gadgeel S, Rodríguez-Abreu D, Speranza G, Esteban E, Felip E, Dómine M et al (2020) Updated analysis from KEYNOTE-189: pembrolizumab or placebo plus pemetrexed and platinum for previously untreated metastatic nonsquamous non-small-cell lung cancer. J Clin Oncol 38(14): 1505–1517

Gettinger S, Borghaei H, Brahmer J, Chow L, Burgio M, Carpeno JDC et al (2019) OA14.04 five-year outcomes from the randomized, phase 3 trials CheckMate 017/057: nivolumab vs docetaxel in previously treated NSCLC. J Thorac Oncol 14(10):S244–S245

Ghisoni E, Imbimbo M, Zimmermann S, Valabrega G (2019) Ovarian cancer immunotherapy: turning up the heat. Int J Mol Sci [Internet] 20(12):2927. [Cited 2020 Aug 30]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6628106/

Gray JE, Villegas AE, Daniel DB, Vicente D, Murakami S, Hui R et al (2019) Three-year overall survival update from the PACIFIC trial. J Clin Oncol 37(15_Suppl):8526–8526

Grob JJ, Gonzalez Mendoza R, Basset-Seguin N, Vornicova O, Schachter J, Joshi A et al (2019) LBA72—pembrolizumab for recurrent/metastatic cutaneous squamous cell carcinoma (cSCC): efficacy and safety results from the phase II KEYNOTE-629 study. Ann Oncol 30:v908

Gunasekaran M, Russo A, Cardona AF, Perez D d M, Lapidus R, Cooper B et al (2020) Exosomal PD-L1 expression as non-invasive biomarker for immune checkpoint inhibitors in non-small cell lung cancer. J Immunol 204(1 Suppl):90.10

Gutzmer R, Stroyakovskiy D, Gogas H, Robert C, Lewis K, Protsenko S et al (2020) Atezolizumab, vemurafenib, and cobimetinib as first-line treatment for unresectable advanced BRAFV600 mutation-positive melanoma (IMspire150): primary analysis of the randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 395(10240):1835–1844

Havel JJ, Chowell D, Chan TA (2019) The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. Nat Rev Cancer 19(3):133–150

He AR, Yau T, Hsu C, Kang Y-K, Kim T-Y, Santoro A et al (2020) Nivolumab (NIVO) + ipilimumab (IPI) combination therapy in patients (pts) with advanced hepatocellular carcinoma (aHCC): subgroup analyses from CheckMate 040. J Clin Oncol 38(4_Suppl):512

Heemskerk B, Kvistborg P, Schumacher TNM (2013) The cancer antigenome. EMBO J 32(2): 194–203

Hellmann MD, Paz-Ares L, Bernabe Caro R, Zurawski B, Kim S-W, Carcereny Costa E et al (2019) Nivolumab plus ipilimumab in advanced non-small-cell lung cancer. N Engl J Med 381(21): 2020–2031

Herbst R, Marinis FD, Giaccone G, Reinmuth N, Vergnenegre A, Barrios C et al (2020a) O81 IMpower110: interim overall survival (OS) analysis of a phase III study of atezolizumab (ATEZO) monotherapy vs platinum-based chemotherapy (CHEMO) as first-line (1L) treatment in PD-L1–selected NSCLC. J Immunother Cancer [Internet] 8(Suppl 1). [Cited 2020 Sept 9]. https://jitc.bmj.com/content/8/Suppl_1/A1.1

Herbst RS, Garon EB, Kim D-W, Cho BC, Perez-Gracia JL, Han J-Y et al (2020b) Long-term outcomes and retreatment among patients with previously treated, programmed death-ligand 1–positive, advanced non–small-cell lung cancer in the KEYNOTE-010 study. J Clin Oncol 38(14):1580–1590

Horn L, Mansfield AS, Szczęsna A, Havel L, Krzakowski M, Hochmair MJ et al (2018) First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. N Engl J Med 379(23):2220–2229

Johnson DB, Reynolds KL, Sullivan RJ, Balko JM, Patrinely JR, Cappelli LC et al (2020) Immune checkpoint inhibitor toxicities: systems-based approaches to improve patient care and research. Lancet Oncol 21(8):e398–e404

Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704

Kudo M, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer DH et al (2020) Updated efficacy and safety of KEYNOTE-224: a phase II study of pembrolizumab (pembro) in patients with advanced hepatocellular carcinoma (HCC). J Clin Oncol 38(4_Suppl):518

Larkin J, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Lao CD et al (2019) Five-year survival with combined nivolumab and ipilimumab in advanced melanoma. N Engl J Med 381(16):1535–1546

Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK et al (2017) Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357(6349):409–413

Lenz H-J et al (2020) Nivolumab plus low-dose ipilimumab as first-line therapy in microsatellite instability-high/DNA mismatch repair deficient metastatic colorectal cancer: clinical update. J Clin Oncol [Internet]. [Cited 2020 Sept 8]. https://ascopubs.org/doi/abs/10.1200/JCO.2020.38.4_suppl.11

Mansfield AS, Aubry MC, Moser JC, Harrington SM, Dronca RS, Park SS et al (2016) Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor

infiltration between paired primary lesions and brain metastases in lung cancer. Ann Oncol 27(10):1953–1958

Marabelle A, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord J-P et al (2020) Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 38(1):1–10

Meeting Library | Maintenance avelumab + best supportive care (BSC) versus BSC alone after platinum-based first-line (1L) chemotherapy in advanced urothelial carcinoma (UC): JAVELIN Bladder 100 phase III interim analysis. [Internet]. [Cited 2020 Sept 9]. https://meetinglibrary.asco.org/record/186872/abstract

Motzer RJ, Rini BI, McDermott DF, Arén Frontera O, Hammers HJ, Carducci MA et al (2019) Nivolumab plus ipilimumab versus sunitinib in first-line treatment for advanced renal cell carcinoma: extended follow-up of efficacy and safety results from a randomised, controlled, phase 3 trial. Lancet Oncol 20(10):1370–1385

Motzer RJ, Tykodi SS, Escudier B, Oudard S, Hammers HJ, McDermott DF et al (2020) Final analysis of the CheckMate 025 trial comparing nivolumab (NIVO) versus everolimus (EVE) with >5 years of follow-up in patients with advanced renal cell carcinoma (aRCC). J Clin Oncol 38(6_Suppl):617

Nghiem P, Bhatia S, Lipson EJ, Sharfman WH, Kudchadkar RR, Brohl AS et al (2019) Durable tumor regression and overall survival in patients with advanced Merkel cell carcinoma receiving pembrolizumab as first-line therapy. J Clin Oncol 37(9):693–702

Nolan E, Savas P, Policheni AN, Darcy PK, Vaillant F, Mintoff CP et al (2017) Combined immune checkpoint blockade as a therapeutic strategy for BRCA1-mutated breast cancer. Sci Transl Med 9(393):eaal4922

Ohyama C, Kojima T, Kondo T, Naya Y, Inoue T, Tomita Y et al (2019) Nivolumab in patients with unresectable locally advanced or metastatic urothelial carcinoma: CheckMate 275 2-year global and Japanese patient population analyses. Int J Clin Oncol 24(9):1089–1098

Patel MR, Ellerton J, Infante JR, Agrawal M, Gordon M, Aljumaily R et al (2018) Avelumab in metastatic urothelial carcinoma after platinum failure (JAVELIN Solid Tumor): pooled results from two expansion cohorts of an open-label, phase 1 trial. Lancet Oncol 19(1):51–64

Paz-Ares L, Luft A, Vicente D, Tafreshi A, Gümüş M, Mazières J et al (2018) Pembrolizumab plus chemotherapy for squamous non–small-cell lung cancer. N Engl J Med 379(21):2040–2051

Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D et al (2019) Durvalumab plus platinum-etoposide versus platinum-etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. Lancet (Lond Engl) 394(10212):1929–1939

Powles T, O'Donnell PH, Massard C, Arkenau H-T, Friedlander TW, Hoimes CJ et al (2017) Efficacy and safety of durvalumab in locally advanced or metastatic urothelial carcinoma. JAMA Oncol [Internet] 3(9):e172411. [Cited 2020 Sept 9]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5824288/

Ready N, Farago AF, de Braud F, Atmaca A, Hellmann MD, Schneider JG et al (2019) Third-line nivolumab monotherapy in recurrent SCLC: CheckMate 032. J Thorac Oncol 14(2):237–244

Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A et al (2019) Updated analysis of KEYNOTE-024: pembrolizumab versus platinum-based chemotherapy for advanced non-small-cell lung cancer with PD-L1 tumor proportion score of 50% or greater. J Clin Oncol 37(7):537–546

Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS et al (2017) Tumor and microenvironment evolution during immunotherapy with nivolumab. Cell 171(4):934

Rini BI, Plimack ER, Stus V, Gafanov R, Hawkins R, Nosov D et al (2019) Pembrolizumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. N Engl J Med 380(12):1116–1127

Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ et al (2015) Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348(6230):124–128

Robert C, Ribas A, Schachter J, Arance A, Grob J-J, Mortier L et al (2019) Pembrolizumab versus ipilimumab in advanced melanoma (KEYNOTE-006): post-hoc 5-year results from an open-label, multicentre, randomised, controlled, phase 3 study. Lancet Oncol 20(9):1239–1251

Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A et al (2016) Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet (Lond Engl) 387(10031):1909–1920

Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H et al (2020) Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 21(1):44–59

Schnipper LE, Schilsky RL (2018) Are value frameworks missing the mark when considering long-term benefits from immuno-oncology drugs? JAMA Oncol 4(3):333–334

Schumacher TN, Schreiber RD (2015) Neoantigens in cancer immunotherapy. Science 348(6230): 69–74

Seidel JA, Otsuka A, Kabashima K (2018) Anti-PD-1 and anti-CTLA-4 therapies in cancer: mechanisms of action, efficacy, and limitations. Front Oncol [Internet] 8:86. [Cited 2020 Sept 8]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5883082/

Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S et al (2017) iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol 18(3): e143–e152

Shah MA, Kojima T, Hochhauser D, Enzinger P, Raimbourg J, Hollebecque A et al (2019) Efficacy and safety of pembrolizumab for heavily pretreated patients with advanced, metastatic adenocarcinoma or squamous cell carcinoma of the esophagus: the phase 2 KEYNOTE-180 study. JAMA Oncol 5(4):546–550

Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N et al (2018) Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. N Engl J Med 378(24):2288–2301

Stewart R, Morrow M, Hammond SA, Mulgrew K, Marcus D, Poon E et al (2015) Identification and characterization of MEDI4736, an antagonistic anti-PD-L1 monoclonal antibody. Cancer Immunol Res 3(9):1052–1062

The Nobel Prize in Physiology or Medicine (2018) [Internet]. NobelPrize.org. [Cited 2020 Sept 6]. https://www.nobelprize.org/prizes/medicine/2018/summary/

Vuky J, Balar AV, Castellano D, O'Donnell PH, Grivas P, Bellmunt J et al (2020) Long-term outcomes in KEYNOTE-052: phase II study investigating first-line pembrolizumab in cisplatin-ineligible patients with locally advanced or metastatic urothelial cancer. J Clin Oncol 38(23): 2658–2666

Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL et al (2017) Adjuvant nivolumab versus ipilimumab in resected stage III or IV melanoma. N Engl J Med 377(19): 1824–1835

Zinzani PL, Lee HJ, Armand P, Johnson N, Brice P, Radford J et al (2019) Three-year follow-up of Keynote-087: pembrolizumab monotherapy in relapsed/refractory classic Hodgkin lymphoma. Blood 134(Suppl_1):240

Zou W, Chen L (2008) Inhibitory B7-family molecules in the tumor microenvironment. Nat Rev Immunol 8(6):467–477

Recent Developments in the Immunotherapeutic Approaches for Cancer Treatment

21

Shiv Bharadwaj, Nikhil Kirtipal, and R. C. Sobti

Abstract

Cancer is basically a class of disorder marked by uncontrolled proliferation of cells which have the potential to interfere with different systems of body like digestive, central nervous, and circulatory systems by releasing hormones. Immunotherapeutic approaches, which involve manipulation or augmentation of specific anticancer immune responses, are now preferably applied in the treatment of these malignancies, and traditional therapeutic approaches are being replaced by the use of agents which potentiate immune effector mechanisms, broadly called "immunotherapy." Cancer immunotherapies are generally classified into two major main classes: active and passive methods. Interventions to augment the immune system of the patient, for example, vaccination or adjuvant therapy, actively promote antitumor effector mechanisms to improve cancer elimination. On the other hand, administration of specific monoclonal antibodies against different tumor antigens and adoptive transfer of genetically-modified specific T cells are currently the most rapidly developing approaches for cancer targeted therapy. In this chapter, we have provided the recent insights on the developed immunotherapeutics and their mechanisms with respect to the type of cancer along with pros and cons of the therapy.

S. Bharadwaj
Department of Biotechnology, Institute of Biotechnology, College of Life and Applied Sciences, Yeungnam University, Gyeongsan, Gyeongbuk, Republic of Korea

N. Kirtipal
Department of Science, Modern Institute of Technology, Rishikesh, Uttarakhand, India

R. C. Sobti (✉)
Department of Biotechnology, Punjab University, Chandigarh, India
e-mail: rcsobti@pu.ac.in

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_21

Keywords

Cancer · Malignant · Immunotherapy · Monoclonal antibodies · Adjuvant · Antitumor · T cells · Vaccination

21.1 Introduction

Cancer is generally known as an assembly of linked diseases. Typically, in all types of cancer, some cells in the body show uncontrolled cell division and proliferate into adjacent tissues or organs. Cancer can initiate or originate at any site in the human body, which is composed of trillions of cells. Usually, the human cells undergo cell division by either mitotic or meiotic cell cycles to produce new cells as requirement in the body. Eventually, cells become old or damaged, removed, and replaced by the new cells. However, under specific conditions, these cells break down the orderly process and attained the characteristics of cancer. Thus, the old and damaged cells survive in the body along with new cells under cancerous conditions, which are not needed in the body. These cells together show further continuous cell division and may form complied cell growths, known as tumors.

Hence, cancer is a genetic disease, i.e., it is associated with the damages in the genes that control the normal cellular functions, especially the cell growth and division process. The changes in the genome or genetic alternations, which can cause cancer, can be inherited to the offspring from their parents. Besides, these genetic changes can also produce in the body of a person's lifetime as a consequence of errors or DNA damages that occur as cell divided or exposed to certain external environmental conditions, respectively. The specific environmental parameters which can induce cancer in the body include chemical substances such as tobacco smoke, xenobiotic compounds, etc., and physical factors like ultraviolet rays from the sun. Each type of cancer in a patient has unique combination of genetic mutations or sometimes specific gene associated with them; these genes are called as oncogenes. Remarkably, as the cancer in the body continues to grow, additional changes produce within the same cancer where different cells may possess different expression of genes or genetic alternations. For example, teratoma tumor which contained several different types of gene expression and lead to formation of various tissue in the formed growth of cells such as hair, muscle, teeth, or bone. In general, cancer cells harbor several genetic alternations such as DNA mutation compared to normal cells. Sometimes, these mutations are not the cause to give rise to cancer but rather produced in the cells by the cancer. Therefore, based on the site of origin or type of tissue that become cancerous, more than 100 types of cancer have been classified and studied in the human body.

21.2 Classification of Cancer by Tissue Types

Generally, based on the site of origin or associated tissue type, cancer can be classified as epithelial cells or mesenchymal cells-based type in the body. Epithelial cells are specialized cells, which line organs, vessels, and cavities in the body. In contrast, mesenchymal cells are unspecialized cells, which hold the capability to differentiate into any other type of cells at any interval in the body. Whilst, a neoplasm is defined as the type of excessive and abnormal growth, known as neoplasia, of tissue. The neoplasm growth is uncoordinated with that of the normal adjacent tissue, and continues in growing abnormally, even in the absence of original trigger (Cooper 1992; Birbrair et al. 2014). This process leads to formation of a mass, may be termed as a tumor (Pugh 2000). International Classification of Diseases, Tenth Revision (ICD-10) categorizes neoplasms under four main groups: (1) in situ neoplasms, (2) benign neoplasms, (3) malignant neoplasms, and (4) neoplasms of uncertain or unknown behavior. Basically, malignant neoplasms are also called as cancers and are the center of oncology. Besides, people often synonymously use the terms tumor and cancer but it is important to mention that all tumors are not cancerous in the body. Broadly, the tumors are classified into main two types, i.e., (a) malignant tumors and (b) benign tumors, as shown in Fig. 21.1.

(a) **Malignant Tumors**
This type of cancer cells have the ability to invade into surrounding healthy tissue or organs and destroy them. Even, this type of cancer can proliferate to distant parts of the body with the aid of bloodstream or lymphatic system; among these tumors, only a few of them are cancerous in nature.

(b) **Benign Tumors**

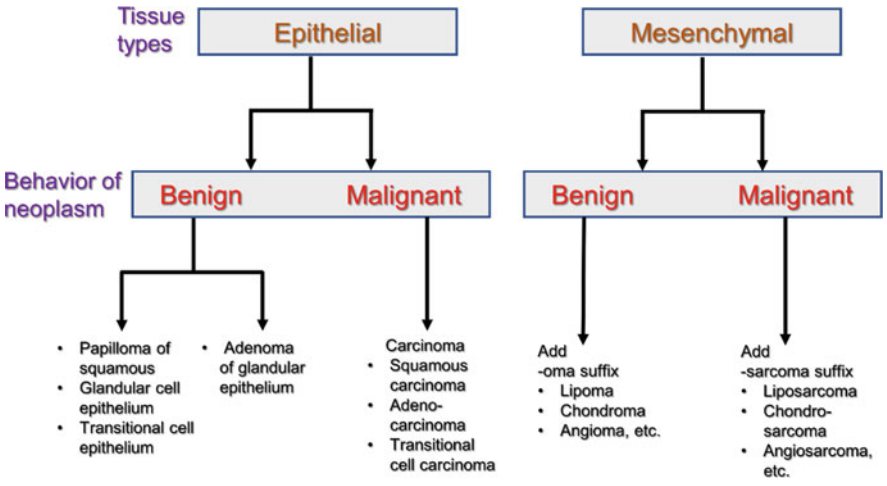


Fig. 21.1 Classification of cancer with respect to cell and tissue types

These types of tumors are a collection of cells that lack the facility to grow uncontrollably and invade neighboring tissues or metastasize. Unlike, malignant tumors, this type of tumor can be removed from the body and usually do not grow back. In contrast to most benign tumors growth in the body, benign brain tumors are established as life-threatening cancer (National Cancer Institute 2017, https://www.cancer.gov/news-events/press-releases/2017/annual-report-to-nation-1975-2014). Typically, these cancers are covered by an fibrous sheath of connective tissue or packed within a epithelium (Martini 2006). Most common benign tumors examples are moles and uterine fibroids.

Furthermore, based on the type of tissue, the international standard for the classification and nomenclature of histologies is the International Classification of Diseases for Oncology Third Edition (ICD-O-3) subdivided the cancer into six major groups, viz.: (1) carcinoma, (2) sarcoma, (3) myeloma, (4) leukemia, (5) lymphoma, and (6) mixed types. These types of cancers can be further grouped based on the cell types such as epithelial cell or a squamous cell as discussed below.

21.2.1 Carcinoma

This type of cancer is of the most common type that occur in epithelial cells; these cells cover both external and internal surface of the body. Hence, carcinomas are broadly separated into two groups: metastatic (the principal cause of cancer-related deaths) and nonmetastatic (Siegel et al. 2016). Traditionally, metastasis is considered to be established in the later stages of cancer progression; however, recent studies have also described metastatic dissemination in early phase of tumor formation (Hosseini et al. 2016). During metastasis, cancer cells under dissemination phase escape from primary tumors and obtain the cellular traits that permit them to travel and inhabit distant organs (Chambers and Werb 2015; Lambert et al. 2017; Gonzalez et al. 2018b). Primary and metastatic tumors have complex ecosystems and are composed of neoplastic cells, extracellular matrix (ECM), and "accessory" nonneoplastic cells, which include resident mesenchymal support cells, infiltrated inflammatory immune cells, and endothelial cells. The dynamic cross cell signaling between accessory cells and cancer cells shapes and fuels the tumor growth. Moreover, a highly specialized microenvironment, formed by tissue architecture during tumor formation, has been characterized by a chronic inflammation and corrupted ECM (Coussens and Werb 2002). Therefore, based on the origin of cancer in epithelial cell type, carcinomas are classified with specific names as discussed below.

21.2.1.1 Adenocarcinoma

This type of cancer occur in the forms of epithelial cells that generate mucus or fluids. Thus, tissue composed of these epithelial cells are also known as glandular tissues; for example, most cancers of the prostate, colon, and breast are caused by adenocarcinomas.

21.2.1.2 Basal Cell Carcinoma

This cancer originates in the basal or lower layer of the epidermis such as skin.

21.2.1.3 Squamous Cell Carcinoma

This cancer, sometimes called as epidermoid carcinomas, occurs in the squamous cells, which are epithelial cells located beneath the outer surface of the skin. Besides, these squamous cells also form the lines of several internal organs, including the lungs, stomach, intestines, kidneys, and bladder.

21.2.1.4 Transitional Cell Carcinoma

The type of cancer, which forms in transitional epithelium, or urothelium tissue, is known as transitional cell carcinoma. As this tissue, composed of several layers of epithelial cells that can be smaller or bigger, is responsible for the generation of linings in part of the kidneys (renal pelvis), bladder, ureters, and a few other organs; thus, some of the cancers of these organs are classified as transitional cell carcinomas.

21.2.2 Sarcoma

Malignant tumors rising in mesenchymal tissue are called sarcomas. These are composed of soft tissue in the body, including tendons, muscle, tissue around joints, fat, lymph vessels, blood vessels, and nerves. Thus, these cancers usually formed in bone and soft tissues, such as muscle, fat, blood vessels, lymph vessels, and fibrous tissue (tendons and ligaments).

21.2.2.1 Osteosarcoma

This tumor is well known as the most common type of cancer in bone. Some of the most common types of soft tissue sarcoma are Kaposi sarcoma, leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma, and dermatofibrosarcoma protuberans.

21.2.3 Myeloma

Myeloma is a cancer that instigates in plasma cells, one of the types of immune cell. The abnormal plasma cells, known as myeloma cells, form in the bone marrow and, thus, causes tumors in all the bones in the body. Sometimes, multiple myeloma grows in the body and is also known as plasma cell myeloma and Kahler disease.

21.2.4 Leukemia

Cancers that instigate in the blood generating tissue of the bone marrow are known as leukemias. These cancers do not produce solid tumors but cause a huge production of abnormal white blood cells (leukemia cells and leukemic blast cells) from the

bone marrow that result in jamming out the normal blood cells. This leads to failure of resistance system in the circulatory system against infections and require transportation of oxygen to its tissue as well as control bleeding. Leukemia is classified into four common types based on how speedily the disease gets worse (acute or chronic) and origin of cancer in the type of blood cell (lymphoblastic or myeloid).

21.2.5 Lymphoma

Lymphoma is a cancer that starts in lymphocytes (B cells or T cells), which are disease-fighting white blood cells and are significant part of the immune system. In lymphoma, abnormal lymphocytes build up in lymph vessels, lymph nodes, and in other organs. There are two main categories of lymphoma: (1) Hodgkin lymphoma—people with this disease produce abnormal lymphocytes, known as Reed-Sternberg cells, which are usually produced by the B cells. (2) Non-Hodgkin lymphoma—a large collection of cancers that occur in lymphocytes. This type of cancers can cultivate quickly or slowly and originate from T cells or B cells.

21.2.6 Mixed Types

21.2.6.1 Germ Cell Tumors

These cancers are defined as tumor that start in the cells, which produce sperm or eggs. These tumors can happen nearly any part of the body and may be either benign or malignant type.

21.2.6.2 Neuroendocrine Tumors

These tumors originate from cells that release hormones into the blood against the response to a signal produce by the nervous system. These tumors, which lead to release of higher-than-normal amounts of hormones in the body, can source several different symptoms. Such tumors in the body may be of benign or malignant type.

21.2.6.3 Carcinoid Tumors

Carcinoid tumors are a category of neuroendocrine tumor. They are slow-growing tumors that usually originate in the gastrointestinal system (mostly in the small intestine and rectum). Carcinoid tumors can spread to the liver or other organs in the body, and may release signals such as prostaglandins or serotonin, triggering carcinoid syndrome.

21.2.6.4 Melanoma

Melanoma is a cancer that starts in cells that develop melanocytes, which are specific cells for the melanin production (pigment which produce skin color). Most melanomas produce on the skin but can also originate in other pigmented tissues, such as eye, in the body.

21.2.6.5 Brain and Spinal Cord Tumors

There are diverse types of brain and spinal cord tumors. These tumors are labeled based on initial location or site of tumor formation or cell type in which they produce in the central nervous system. For example, an astrocytic tumor starts in star-shaped brain cells known as astrocytes, which provide support to keep nerve cells healthy. Brain tumors can be benign or malignant.

21.3 Overview on Tumor Microenvironment and Immune System

Cancers are not a form of distorted cells but relatively a new organ formed by the several nonmalignant cells containing a large part of the tumor mass, which have become disobedient and lack the ability to sustain a communication bridge to preserve homeostasis in the tissue architecture (Bissell and Radisky 2001). The cells included herein are adipocytes, fibroblasts, vascular endothelial cells, pericytes, and immune cells (Hanahan and Coussens 2012). Cancer-associated inflammation, which exists at various stages of tumorigenesis, contributes to epigenetic alternations, genomic instability, stimulation of angiogenesis, induction for cancer proliferation, promotion of cancer anti-apoptotic pathways, and eventually lead to cancer dissemination (Hanahan and Weinberg 2011). Thus, recent studies have demonstrated that immune cells are the main players in cancer-related inflammation (Gonzalez et al. 2018a). Similar to organogenesis during the development, tumor and stromal cells developed together, and the cellular dialogue among the various components results in a constant phenotypic and functional plasticity. Dynamic reciprocal interaction between cells and microenvironment is directed via junctions and receptors along with a plethora of signals released from the multiple cell types enclosed in a three-dimensional extracellular matrix (ECM). This comprises cytokines, proteoglycans, glycoproteins, and growth factors, organized with ECM-remodeling enzymes, deliver both appropriate signals and structural support (Pickup et al. 2014). The interruption of tissue homeostasis generates dynamic fluctuations in the cellular metabolism and functional characteristics in both immune and stromal cells (Buck et al. 2017); this highly operated system institutes the tumor microenvironment (TME) (Galli et al. 2020). Interestingly, the immune cells trapped in the TME essentially contribute in tumorigenesis, suggested to have tumor-promoting or tumor-antagonizing functions. The tumor-antagonizing immune cells primarily consist of effector T cells (effector $CD4^+$ T cells and $CD8^+$ cytotoxic T cells), M1-polarized macrophages, N1-polarized neutrophils, dendritic cells (DCs), and natural killer (NK) cells (Lei et al. 2020). Individual human tumors arise through a combination of genetic and epigenetic changes that facilitate immortality, but at the same time create foreign antigens, the so-called neo-antigens, which should render neoplastic cells detectable by the immune system and target them for destruction. Nevertheless, although the immune system is capable of observing differences in protein structure at the atomic level, cancer cells manage to escape immune recognition and subsequent destruction. For instance, tumor-antagonizing immune incline to

target and destroy the cancer cells during initial stage of tumorigenesis; however, cancer cells appear to escape from immune recognition and subsequent destruction, and even block the tumor-antagonizing immune cells cytotoxic function by numerous mechanisms. To achieve this, tumors develop multiple resistance mechanisms, including local immune evasion, induction of tolerance, and systemic disruption of T cell signaling. Moreover, in a process termed immune editing, immune recognition of malignant cells imposes a selective pressure on developing neoplasms, resulting in the outgrowth of less immunogenic and more apoptosis-resistant neoplastic cells (Teng et al. 2015). Also, TEM contained sufficient number of tumor-promoting immune cells, such as mainly from myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) (Lei et al. 2020). The ability of immune cell to invade the TEM provides opportunities for new therapeutic approaches against cancer, namely utilizing the immune cells to fight the cancer cells (Lei et al. 2020).

The immune system contains several forms of soluble bioactive molecules, proteins, cytokines, and cells that together generate the multifaceted system of biochemical processes; this network assists in the recognition as well as defends against the foreign entities like nonself protein or antigens (Murphy et al. 2008a, b). To maintain and provide the host's homeostasis at equilibrium, the immune system is grouped into two forms for induction of immune responses, i.e., (a) innate and (b) adaptive (Fig. 21.2) (Murphy et al. 2008a, b). Furthermore, the immediate and nonspecific immune responses are categorized as innate because of their fast-acting nonspecific feedback against the foreign bodies, such as allergenic antigens, pathogenic microbes, or molecules/non-self-proteins (Murphy et al. 2008a, b; Kumar et al. 2011). Also, innate immunity exists for short interval and lacks capability to store an immunological memory; however, it still holds the capability to differentiate between "self" and "nonself" or foreign groups of antigens via special receptors like toll-like receptors (TLRs) and pathogen associated molecular patterns (PAMPs) (Kumar et al. 2011). For example, Toll-like receptor 7 (TLR7 receptor) assists in the identification of single stranded RNA as well as suppresses the activation of Tregs, which is advantageous in the tumor environment (Adams et al. 2015; Dominguez-Villar et al. 2015). Other molecules such as cytokines and complement proteins show the defense mechanisms in the host for immediate protection by the innate immunity (Murphy et al. 2008a, b). Notably, cytokines have various roles conditional to the secreted microenvironment, the secretion source of cells, the receptor location to which they bind to, and the activated signaling pathways following their binding with the receptor (Dinarello 2007). Besides, complement proteins exist in three major signaling pathways, which are induced by alternative, classical, and the lectin pathways; all the pathways result in activation of complement proteins. Following, the activated complementary proteins function in opsonization, stand as chemoattractant for supplementary immune cells, and mediate cell/pathogen (only cancer/tumor) demise by development of membrane attack multifaceted for lysis (Wills-Karp 2007). Besides, the major players in cell-mediated innate immune responses include phagocytes and natural killer (NK cells) (Sun and Lanier 2009). These phagocytes, i.e., neutrophils, monocytes, and macrophages, enable immediate host defense by engulfing cells that express non-self-antigens or

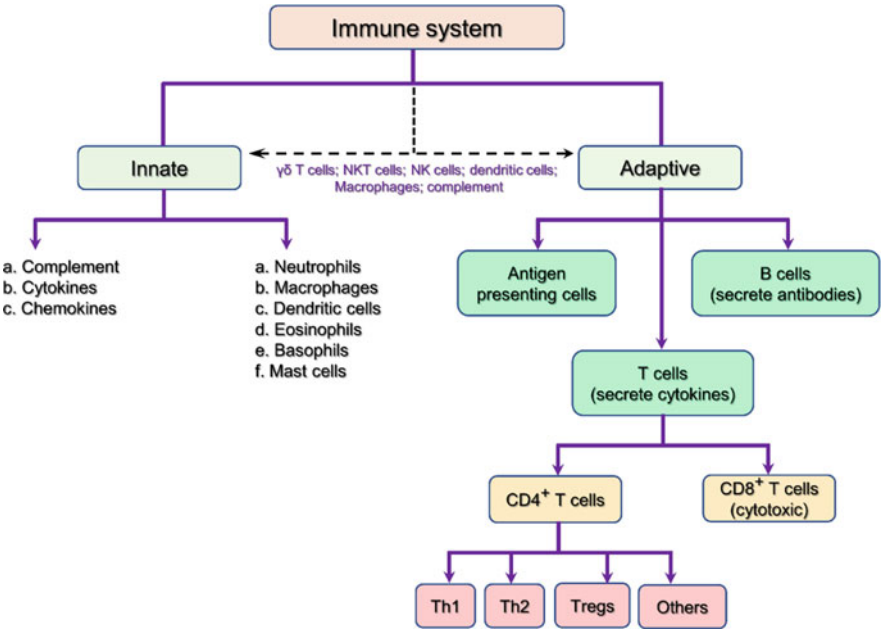


Fig. 21.2 Overview on the immune system, i.e., innate and adaptive immunity

altered self-antigens and destroy them with lysosomal enzymes (Fig. 21.2) (Sun and Lanier 2009). Whilst, NK cells converse immune protection through major histocompatibility complex I (MHC class I) proteins; these molecules universally expressed on the cell membrane of all the nucleated cells (Sun and Lanier 2009). Moreover, these cells also secrete granzyme and perforin to encourage cell apoptosis that have altered MHC class I expression if the cell has been compromised (Sun and Lanier 2009). Other cells, viz. eosinophils, basophils, and mast cells, release inflammatory mediators such as chemotactic leukotrienes that also contribute in the cellular innate immunity by engaging more immune cells at the site of inflammation/injury (Fig. 21.2) (Murphy et al. 2008a, b). In contrast to innate immune responses, the adaptive immunity comprises the advance of immunological memory because of specific forms of immune responses directing the antigens (Fig. 21.2) (Murphy et al. 2008a, b). This form of immunity happens over time and is not featured as being rapid immune response due to naive lymphocytes like the T and B cells, growing the ability to distinguish and develop into either effector T cells or antibody-secreting B cells (Fig. 21.2) (Murphy et al. 2008a, b). Furthermore, T cells are of two types present in the immune system, which are differentiated by their respective T cell receptor type, i.e., $\alpha\beta$ T cells and $\gamma\delta$ T cells (Fig. 21.2) (Chien et al. 1996). However, only a minor subset of T cells are marked as $\gamma\delta$ T cells and able to recognize "nonself" molecules through pattern recognition, thereof, these cells have no requirement for the MHC-mediated antigen presentation (Fig. 21.2) (Chien et al. 1996). Whilst, $\alpha\beta$ T cells are further divided into two other subsets called cluster of

differentiation 4 $(CD4)^+$ T cells and cluster of differentiation 8 $(CD8)^+$ T cells (Fig. 21.2) (Koretzky 2010; Luckheeram et al. 2012). Development of naive $CD4^+$ T cells into effector $CD4^+$ T cells comprises co-stimulation among major histocompatibility complex II (MHC class II) that expressed only antigen-presenting cells such as B cells, dendritic cells, and macrophages as well as T cell receptor on the naive $CD4^+$ T cells (Fig. 21.2) (Koretzky 2010; Luckheeram et al. 2012). Depending on the cytokine milieu incidence in the microenvironment and the occurrence of other certain transcription factors at the interval of secretion of costimulatory signal, the $CD4^+$ T cells can discriminate into various subsets of effector T cells like Tregs or T helper 1 (Th1) cells and T helper 2 (Th2) cells (Fig. 21.2) (Luckheeram et al. 2012). Interestingly, these subsets can further produce and secrete specific cytokines that accordingly modulate the immune responses (Luckheeram et al. 2012). For example, Th1 cells produce IFN-γ and interleukin-2 (IL-2), and play a role in autoimmunity. Notably, Th2 cells release interleukins 4, 5, 10, 13, and 31 (IL-4, IL-5, IL-10, IL-13, and IL-31), and control the immune responses that are related to the allergic diseases as well as extracellular pathogens (Luckheeram et al. 2012). Likewise, Tregs assist in the reduction of inflammation through IL-10, interleukin-35 (IL-35), and transforming growth factor-beta (TGF-β) production (Luckheeram et al. 2012). Like NK cells of innate immunity, maturation of naive $CD8^+$ T cells into effector cytotoxic T cells depends on MHC class I (Koretzky 2010). The respective MHC class I molecules expressed on surface of nucleated cells can identify and bind to peptides of nonself antigens and abnormal or altered self-antigens (Murphy et al. 2008a, b; Koretzky 2010). Therefore, $CD8^+$ T cells with the aid of specific T cell receptor docked with the antigen/MHC class I multiplexes on the antigen-presenting cells, viz. target cells, which results in the secretion of granzymes and perforin from $CD8^+$ T cells followed by demises of target cells (Koretzky 2010). Both forms of T cells, i.e., $CD4^+$ and $CD8^+$ cells, express other cell surface markers like CTLA-4 and CD28 that contribute in stimulating or preventing the naive T cells, respectively via docking to CD80/CD86 on antigen-presenting cells during co-stimulatory signaling (Podojil and Miller 2009). Remarkably, programmed cell death protein 1 (PD-1) marker on the T cell surface, which binds with the ligands PD-L1 and PD-L2 expressed on antigen-presenting cells, also obstructs T cell activation (Podojil and Miller 2009). Notably, certain cancer cells can also provoke PD-L1 expression as a mechanism to defeat and escape the immune system (Podojil and Miller 2009).

For the development and activation of B cells, antibody-secreting effector functions can be triggered by cell-independent and T helper cell-dependent mechanisms resultant in a wide range of antibodies, which are explicit for the kind of induced immune response (Janeway Jr et al. 2001). Commonly, antibodies are also denoted as immunoglobulins (Ig). All the antibodies consist of two domains: (i) a fragment antibody-binding (Fab) domain which can bind to various antigens and (ii) a fragment crystallizable (Fc) domain which can dock to its respective Fc receptors on effector cells to facilitate effector functions like antibody-dependent complement cytotoxicity (ADCC) (Schroeder Jr and Cavacini 2010). Whilst, all naive B cells prompt membrane-bound antibodies, i.e., IgM and IgD (Schroeder Jr and Cavacini 2010), several other antibody isotypes, like IgA, IgG, and IgE, are also

formed by instant and long-term memory plasma cells by immunoglobulin class switching, affinity maturation, and somatic hypermutations (Schroeder Jr and Cavacini 2010). Moreover, among these antibody isotypes, certain antibodies like IgA and IgG also possess different subsets that can be further characterized as isotypes (Schroeder Jr and Cavacini 2010). These numerous antibody isotypes and subsets can perform distinct functions that are executed under various conditions (Schroeder Jr and Cavacini 2010). In general, however, antibodies function to deactivate the antigen by docking to it, initiate the ADCC mechanism, perform networking with various components of the classical complement pathway to express complement dependent cytotoxicity (CDC), and conjugating with antibody receptors on specific cells to stimulate their effector functions. Similar to NK cells of innate immunity, the adaptive immune responses also possess their own version of NK cells, called as NKT cells. These cells hold properties of both the NK and the T cells due to the expression of natural killer cell-associated surface marker NK 1.1 and T cell receptors (TCRs) (Terabe and Berzofsky 2008). But, some of TCR in NKT cells can vary from the normal TCR; thus, characterizing them as invariant NKT cells (Terabe and Berzofsky 2008). These NKT cells can have ability to identify and bind with self or nonself lipids/glycolipids by expression of CD1d receptor on antigen-presenting cells and further release several cytokines like IL-12 and IFN-γ for initiation of other immune response (Terabe and Berzofsky 2008). Furthermore, there is another form of antibody known as "cytophilic antibody," which arms macrophages and empowers them to identify, bind, and ultimately destroy a tumor cell (Mitchell et al. 1973). In addition to T-cell-mediated immune responses, macrophage-mediated immunity controlled by T-lymphocytes, but also involving the secretion of nonthymus dependent or "bursal dependent" lymphocyte, namely, antibody. Attachment apparently happens at the Fc domain of the antibody, with the Fab part at the other end of the molecule permitted to identify and attach to antigens. The macrophages role has not been defined in the (potential) rejection of tumors in the human, but recent study discovered cytophilic antibodies in the patients serum diagnosed with melanoma and leukemia (Mitchell et al. 1973). Also, a related antibody, which enables cytotoxicity of tumor cells facilitated by lymphocytes, seems to be another variation of cytophilic antibody arming unsensitized non-T-lymphocytes in spite of macrophages. The diagram in Fig. 21.3 schematizes the interaction of three principal cells involved in the destruction of a tumor (Mitchell 1976). The T-lymphocyte regulated macrophage activity through mediator "lymphokines" that trigger macrophages in a non-specific way (Mitchell 1976; Mitchell et al. 1977). The macrophage also released mediators, such as "lymphocyte activating factor" (LAF) (Gery and Waksman 1972), stimulated the T cells, which showed a bidirectional interaction between lymphocytes and macrophages.

It was also observed that there were also inhibitors produced by each type of cell that permit regulation of the function of the other, not simply activation. During the course of mechanism non-specifically activated macrophage destroy the tumor cells, which may be complemented by specific arming by cytophilic antibodies released from B cells. Experimentally, it was observed that T-lymphocytes can kill tumor

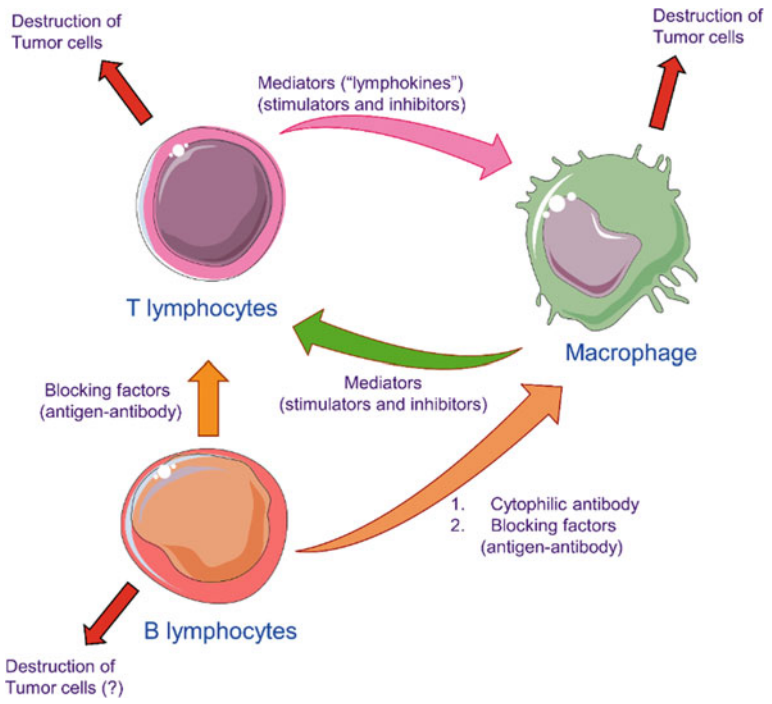


Fig. 21.3 Interaction of lymphocytes and macrophages in tumor destruction

cells directly in vitro; however, under in vivo experiment, 10% of the cells were activated to kill tumor cells where 90% of the cells were identified as macrophages, suggested that macrophages are the major effector cells to kill the tumor cells (Mitchell 1976).

21.4 Background of Cancer Immunotherapy

Cancer immunotherapy, occasionally known as immuno-oncology, is the artificial stimulation of the immune system in the treatment of cancer and enhancement of immune system's natural ability against the disease. This approach can be applied in two different ways: (1) stimulating patient's immune system to act effectively against cancer cells, and (2) providing patient immune system components such as artificial immune system proteins. Additionally, some immunotherapy types are also called as biologic therapy. Recently, immunotherapy has become a significant therapeutic approach against various types of cancer. Cancer immunotherapy exploits the element that cancer cells regularly expressed as tumor antigens or molecules, e.g., carbohydrates, on their surface that can be noticed by the antibody

proteins from the immune system to bind with them. Generally, normal antibodies dock with external pathogens but the designed immunotherapy antibodies assist the immune system to mark and recognize the malignant cells for their inhibition or destruction by binding with the tumor antigens.

In 2018, American immunologist James P. Allison and Japanese immunologist Tasuku Honjo received the Nobel Prize in Physiology or Medicine on the discovery of cancer therapy through inhibition of negative immune regulation. Although, it has been known for several years that the immune system plays a chief role in neoplastic growth and control, since patients with immunosuppressed disabilities have higher risk for cancer, and impulsive regression of numerous forms of malignant tumors is an occasional but well documented phenomenon-occurring in nearly 1 in every 60,000 to 100,000 cancer cases (Challis and Stam 1990; Kucerova and Cervinkova 2016; Chida et al. 2017). Although several cases malignant growths regressing or vanishing after an infectious and/or high febrile episode were reported from ancient Egypt till early eighteenth century in Europe, but the scientific origin for efforts at modifying the immune system for cancer treatment discovered its modern roots solitary in the second half of the eighteenth century, when histologic validation of a malignancy became possible. Around 135 years ago the German physicians Busch (1868) and Fehleisen (1882) independently perceived deterioration of tumors in cancer patients on subsequent accidental infections by erysipelas. In 1868, Busch was the first to purposely infect a cancer patient with erysipelas, and he observed reduction of the malignancy. Fehleisen (1882) recurrent this treatment in 1882 and he also ultimately recognized *Streptococcus pyogenes* as the causative factor of erysipelas (Fehleisen 1882; Oelschlaeger 2010). In 1891, William Coley, an American surgeon, of the Bone Tumor Service at Memorial Hospital in New York, tracked up on his own autonomous observation of a long-term deterioration of sarcoma afterward an erysipelas infection by opening a 43-year-old project related to heat inactivated bacteria ("Coley's toxins") injection into patients suffered with inoperable cancers (Coley 1991). He testified a substantial number of reversions and treatments in more than 1,000 patients, numerous or furthermost with sarcomas, and the procedure started gaining wide acceptance and notoriety (Coley 1910). Also, the establishment of chemotherapy and radiation therapy further contribute to lack of interest using this kind of therapy in cancer treatment (Parish 2003). Coley's principles have been revealed to be correct, and the usage of bacteria lastly found sound validation in 1976 when Morales et al. (1976) recognized the efficiency of the bacterium *Bacillus Calmette-Guérin* (BCG) against superficial bladder cancer; the foundations for this clinical trial contain a 1959 study by Old et al. which display the anti-tumor properties of BCG in a mouse model (Old et al. 1959). Besides, Old also completed extensive research on additional CI-related subjects and later discovered the tumor necrosis factor in 1975 (Carswell et al. 1975). Because of their foundational discoveries and lifelong devotion to the field, Coley and Old have each been

stated to as the "Father of Immunotherapy" (Oiseth and Aziz 2017). Even viral infections were considered to impose anti-cancer effect as far back as 1904 when George Dock at the University of Michigan pronounced a 42-year-old woman with acute leukemia who experienced a temporary reduction following a presumed influenza infection in 1896 (Larson et al. 2015).

Burnet (1957, 1970) and Thomas and Lawrence (1959) were the first to suggest the concept that immune system have the ability to identify and destroy nascent "non-self" malignant cells in their cancer immunosurveillance hypothesis. However, their concept was not recognized initially but it is now measured as component of cancer immunoediting; herein, the surveillance system can govern or "shape" the immunogenicity of the tumor cells, which were not eradicated initially (Dunn et al. 2002). The immunoediting procedure has been properly divided into three main phases: elimination, equilibrium, and escape. The elimination phase denotes to the initial impairment and possible devastation of cancer cells by the innate immune system, trailed by exhibition of the tumor antigens in the cellular debris for dendritic cells which then display them to T cells and thus, generate tumor-specific $CD4^+$ and $CD8^+$ T cells. These immune responses assist in the destruction of remaining tumor cells if abolition is complete. The equilibrium phase happens when any tumor cells escape the initial eradication attempt but lack the ability to grow, being preserved in a state of evenness with the immune cells. Whilst, during escape phase, cancer cells nurture and metastasize because of lack in mediation by the immune system. The dynamic that occurs between tumor antigens and the immune system is a phenomenon identified relatively recently, since it was only in 1991 that van der Bruggen and colleagues first described the existence of a human tumor antigen detected by T cells (van der Bruggen et al. 1991). Remarkably, they clone the melanoma antigen-encoding gene (MAGE), which expresses an antigen detected by cytotoxic T cells. This discovered not only the evidence that immune system can seek and destroy tumor cells but also provided the first detection of a molecular target. Thus, the capability of cancer cells to escape immune surveillance-based destruction has been projected as the eighth hallmark of cancer (Hanahan and Weinberg 2011). It is now well known that even before cancer metastasize, it can alter the systemic environment by varying hematopoiesis and the tissue parenchyma in the organs at distant sites, thereby establishing "pre-metastatic niches" (McAllister and Weinberg 2014). While a few cancer immunotherapies have had significant successes in altering these tumor microenvironments, the lack of MHC class I expression in tumor displays a main challenge in immunotherapy-based treatment (Garrido et al. 2016). Besides, the intrinsic immunological aptitude of an individual to fight cancer has been termed as the "cancer–immune set point," and is altered by a complex set of factors such as the host, tumor, and environmental factors (Chen and Mellman 2017).

21.5 Classification of Cancer Immunotherapy

Although, potential role of immune system to halt or as treatment against cancer has been recognized over 100 years ago, but actual therapeutic approach as cancer immunotherapy has become a realism only in the last two decades. At present, two broad approaches are currently in advancement for cancer immunotherapy, i.e., (1) agents that directly target the cancer cells and (2) agents that stimulate immune cells to cause demolition of cancer, as depicted in the Fig. 21.4 (Sathyanarayanan and Neelapu 2015).

21.5.1 Antibody-Based Immunotherapy

Specific identification and removal of malignant cells or pathogens by antibodies was projected over a century ago. Recent advancement in science has already focused on development of monoclonal antibodies (mAbs) that directly target the cancer. Although Paul Ehrlich was the first to propose the "magic bullet hypothesis" in 1897 (Ehrlich 1906), the application of antibodies as "magic bullets" became achievable only following the establishment of the hybridoma technology by Kohler

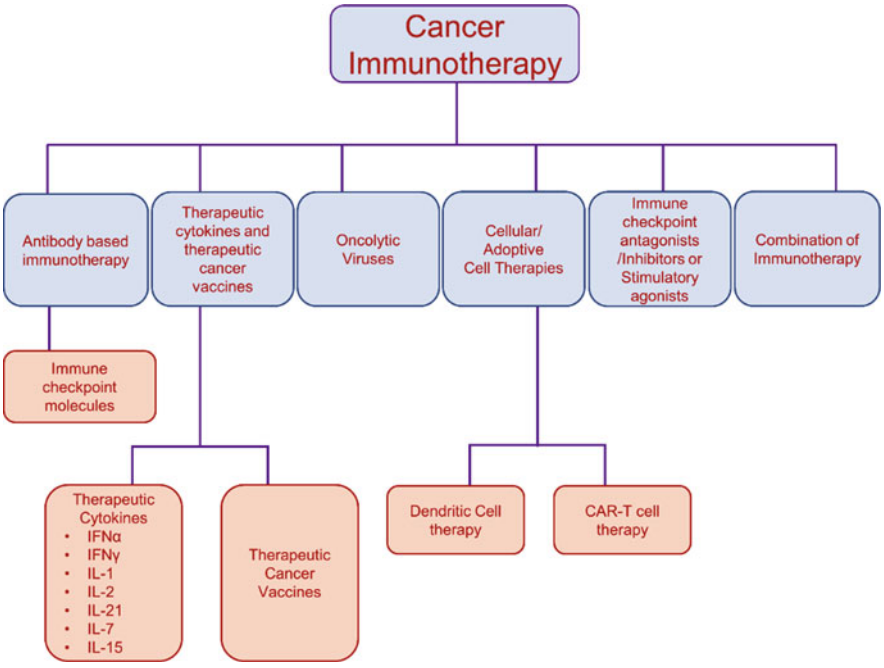


Fig. 21.4 Types of cancer immunotherapy agents broadly categorized into those that target the cancer and those that activate immune cells to kill the cancer along with their respective sub-types of agents within each group (Sathyanarayanan and Neelapu 2015)

and Milstein in 1975 (Kohler and Milstein 1975). The US Food and Drug Administration (FDA) permitted the first monoclonal antibody, i.e., rituximab, for the cancer treatment, in 1997 (McLaughlin et al. 1998). In subsequent years, several monoclonal antibodies were approved against various cancer treatment, including breast cancer, colon cancer, B-cell malignancies, and others.

The "naked" monoclonal antibodies like rituximab and trastuzumab encouraged apoptosis tumor cell death through both Fc dependent and independent mechanisms (Weiner et al. 2010). The Fc dependent mechanisms comprise antibody-dependent cell-mediated cytotoxicity (ADCC) facilitated by NK cells and macrophages, antibody-dependent cellular phagocytosis (ADCP) regulated by macrophages along with complement-dependent cytotoxicity (CDC). The Fc independent mechanisms contain initiation of direct apoptosis after antibodies binding to their receptors or by inhibiting the receptor-ligand interactions; for instance, growth factor signaling mediated through cell receptors like Human epidermal growth factor receptor 2 (HER2) on breast cancer cells (Sathyanarayanan and Neelapu 2015). Besides, application of naked monoclonal antibodies irrespective of application as lone or combination with traditional chemotherapy therapeutics have enhanced the inclusive response rates, complete reduction rates, and progression-free and total survival in multiple cancers such as colon cancer, breast cancer, lymphomas, and others (Vogel et al. 2002; Cheson and Leonard 2008; Van Cutsem et al. 2009; Weiner et al. 2010). To further advance their efficacy, monoclonal antibodies were also functionalized with radioisotopes like Yttrium-90 or Iodine-131 to produce radioimmunotherapy agents (Kraeber-Bodere et al. 2014) or cytotoxic agents such as emtansine or monomethyl auristatin E to generate antibody–drug conjugates (Younes et al. 2010; Zolot et al. 2013). Such functionalized monoclonal antibodies enabled targeted delivery of radioisotopes and cytotoxic agents at the tumor and were testified with improved therapeutic function against corresponding naked monoclonal antibodies. Additionally, radioimmunotherapy agents can also trigger tumor destruction via "crossfire" effect in the non-targeted cancer cells in the vicinity (Sathyanarayanan and Neelapu 2015). Extra strategy to mark the tumor is the application of immunotoxins where an immune molecule like cytokine is functionalized to a toxin. The cytokine attaches to its corresponding receptor located on the cancer cell surface and send the toxin into the cell after receptor-mediated endocytosis. For example, Denileukin diftitox, an immunotoxin, has been approved by the FDA against recurrent cutaneous T-cell lymphoma (Olsen et al. 2001). Herein, diphtheria toxin is functionalized with Interleukin-2 (IL2) which specifically binds with CD25 receptor on the malignant cells; thereby, diphtheria toxin prevents intracellular protein synthesis and results in cell death after its transportation into the cell. In summary, naked monoclonal antibodies and monoclonal antibodies functionalized conjugated to radioisotopes or cytotoxic agents and immunotoxins have been established with significant efficacy against multiple cancers (Sathyanarayanan and Neelapu 2015). Additionally, to mark novel molecules, advancement in antibody-engineering methods and functionalization to novel radioisotopes and cytotoxic agents are anticipated to further improve the beneficial efficacy of monoclonal antibodies as potential treatment for the cancer.

21.5.1.1 Antibodies As Immune Checkpoint Molecules

In 1996, Leach, Krummel and Allison reported that cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-inhibiting monoclonal antibodies (mAbs) in the treatment of tumor in animal models (Leach et al. 1996); these mAbs later popularly known as "immune checkpoint inhibitors" (ICIs). Recently, mAbs have received much consideration because of being comparatively less toxic by comparison to conventional cancer therapies, easy method for their preparation and administion against other types of cancer immunotherapeutic, and hold potential in widespread applications. The approved mAbs for clinical use target either CTLA-4, PD-1, or PD-L1, which "inhibit the negative blocking" of T cells, with a consequential boost in the immune response for the treatment of cancers. Assays of PD-L1 protein countenance by immunohistochemistry are applied to limit the application of anti-PD-L1 antibody against tumors, but it is an deficient measurement exercise due to lack of calibration methods, and sometimes, it can be problematic to distinguish PD-L1-positive tumor cells against other PD-L1-positive cells in the TME (Mino-Kenudson 2016). Moreover, immunohistochemistry showed lower sensitivity compared to studies counting PD-L1 mRNA expression (Ritprajak and Azuma 2015). Anti-PD-1 and anti-PD-L1 antibody approaches are currently the most studied ICIs due to less severe toxicity, or high-grade "immune related adverse effects" (irAEs), against anti-CTLA-4 antibody treatments (Robert et al. 2015; Champiat et al. 2016; Michot et al. 2016; Haanen et al. 2017; Kroschinsky et al. 2017). A wide range of adverse effects have been associated with single or multiple drug regimens, dosage concentration, and treated types of malignancies. The most common side effects are fatigue, diarrhea/colitis, dermatologic and mucosal toxicities, and hepatotoxity. Corticosteroids or other immunomodulators are known to reverse nearly all the toxic effects of these drugs (Champiat et al. 2016; Michot et al. 2016; Haanen et al. 2017; Kroschinsky et al. 2017). However, pneumonitis, an uncommon but possibly severe complication triggered by these drugs, may cause deaths (Naidoo et al. 2017). Besides, these drugs have shown powerful side effects in six healthy volunteers during phase I trial such as provoked cytokine storm associated with multiorgan failure and later resuscitation in the intensive care unit (Suntharalingam et al. 2006). Recently, less toxic antibodies against checkpoint inhibitors have been developed and approved by the FDA for six malignancies in advanced stages, i.e., head and neck cancer, lung cancer, melanoma, renal cell carcinoma, urothelial cancer, and Hodgkin's lymphoma along with several other tumor types being explored in clinical trials (Burstein et al. 2017; Iwai et al. 2017). Besides, some of these trials are applying specific antibodies to control the function of most recently discovered inhibitory and co-stimulatory checkpoints. It is important to note that not all immune checkpoint or immunomodulatory molecules formed as receptor or ligand. Sometimes, these molecules are expressed by the cell in free soluble form, like indoleamine 2,3—dioxygenase (IDO1), an enzyme formed by some stimulated macrophages and also overexpressed in several tumors (Mbongue et al. 2015).

21.5.2 Therapeutic Cytokines and Therapeutic Cancer Vaccines

Therapeutic-based alternation of the cytokine environment is a potential approach in cancer immunotherapy. In addition, regulation of cytokines can straight affect tumor cells, results in apoptosis and blockage of proliferation (Wagner et al. 2004; Lee and Margolin 2011). Alternately, vaccine development against the cancer is a developing field where it either treats present cancer or averts growth of cancer; Vaccines used in the treatment of existing cancers are called therapeutic cancer vaccines. Besides, sometimes vaccines are "autologous," being generated from samples collected from the patient, and are explicit to that patient only.

21.5.2.1 Therapeutic Cytokines

Cytokines are messenger molecules that allow immune system cells to communicate with one another to provide a robust, coordinated, but self-limited feedback against the target antigen. While several forms of signal communication in the immune system occur via direct cell-cell interaction, or the release of cytokines to permit the rapid proliferation of immune signaling in a multifaceted and competent manner. The growing attention over the past two decades in connecting the immune system to eliminate cancer has been attended by heightened efforts to describe cytokines and exploit their massive signaling networks in the development of treatment against cancer (Lee and Margolin 2011). Cytokine plays dual role in the immune signaling where some cytokine contribute in tumor progression and other with antitumor effect (Fig. 21.5). Thus, cytokines secreted by inflammatory/immune cells or tumor can either exert antitumor effects or promote tumor cell survival and development. Chronic inflammation grows through the action of numerous inflammatory mediators, such as TNF-α, IL-6, and IL-17, and results in elimination of antitumor immunity and augmented tumor progression (Fig. 21.5) (Lin and Karin 2007). However, TNF-related apoptosis-inducing ligand (TRAIL) via direct initiation of tumor cell apoptosis, IL-10 by anti-inflammatory effects, and IL-12 through initiation of CTLs and NK cells and production of cytotoxic mediators, can cause tumor destruction or suppression. Interestingly, multiple actions of TGF-β, i.e., cytotoxic in colon cancer cells, and with both positive and negative roles in the TME have been studied (Fig. 21.5) (Lin and Karin 2007). Whilst cytokines directly excite immune effector cells and stromal cells located at the tumor site and increase tumor cell identification by cytotoxic effector cells. Recently, several cytokines, such as GM-CSF, IL-7, IL-12, IL-15, IL-18 and IL-21, enter clinical trials for the patients diagnosed with advanced cancer. Besides, preclinical studies supported the nullification of oppressive cytokines, such as IL-10 and TGF-β in promoting anti-tumor immunity. Additionally, advances in adoptive cell therapy have been developed with the application of cytokines to generate highly precise environment for the development of anti-tumor T cells under in vitro conditions (Lee and Margolin 2011). There are several types of clinically approved permitted recombinant cytokines in immunotherapy against various cancers; some additional cytokines are presently in clinical development. Some of the well-established cytokines are discussed below.

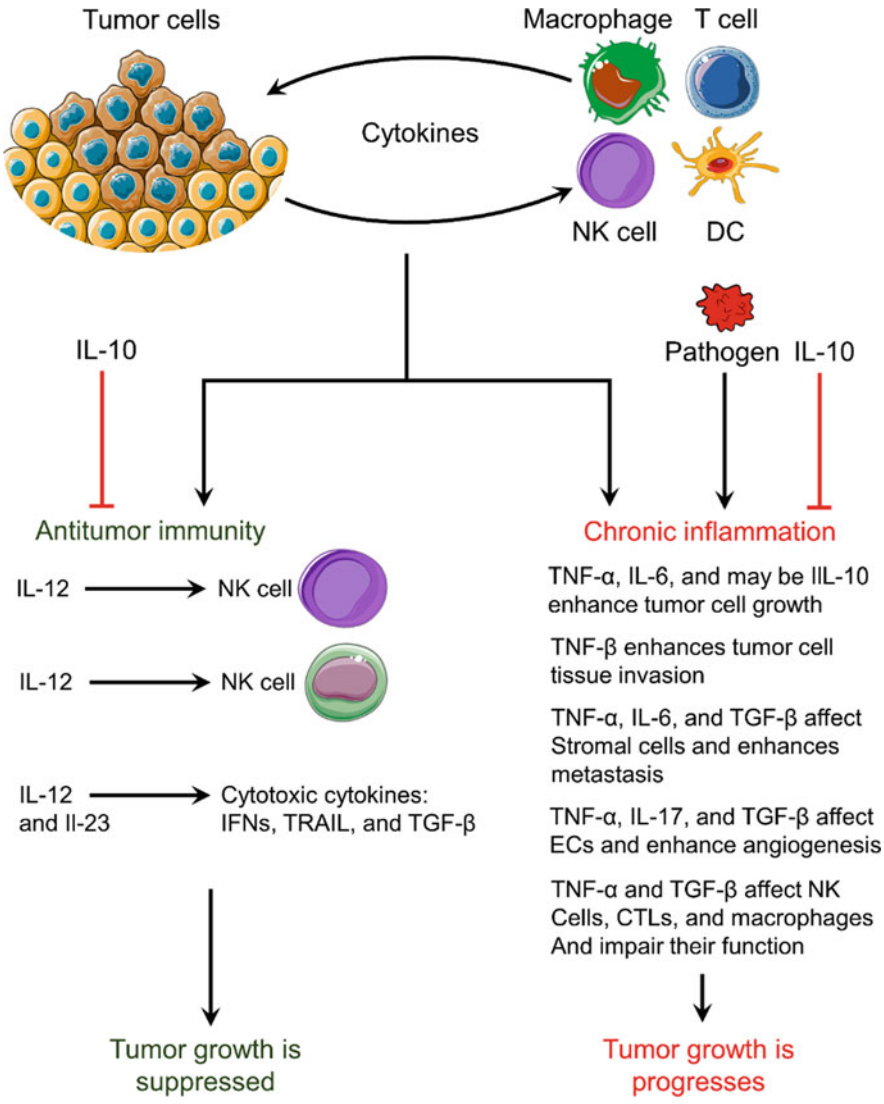


Fig. 21.5 Outcomes produced from the interaction between the tumor cells and infiltrating inflammatory and/or immune cells in the TME (Lin and Karin 2007)

Clinical Application of Interferon Alfa (IFNα)

IFNα was the first cytokine permitted in the treatment of human cancer, i.e., hairy cell leukemia (HCL), in 1986, and following evaluation of several treatment regimens, high-dose IL-2 (HDIL-2) was permitted for metastatic renal cell carcinoma (mRCC) treatment, in 1992, and metastatic melanoma (MM) in 1998. Clinically applied recombinant IFNα formulations occur in three isoforms, viz. alfa-2a, alfa-2b, and alfa-2c. IFNα and Peginterferon alpha 2b are accepted as adjuvant

treatment in patients with fully resected stage III or IV high-risk melanoma, as first-line of treatment in patients with mRCC (alfa-2a, and alfa-2b in mixture with bevacizumab), follicular lymphoma (alfa-2b), AIDS-related Kaposi's sarcoma (alfa-2b), chronic myelogenous leukemia (Philadelphia chromosome-positive alfa-2a), condyloma acuminata (alfa-2b), HCL (alfa-2a, alfa-2b), and cervical intraperitoneal neoplasms (alfa-2b) (Gutterman et al. 1980; Kirkwood and Ernstoff 1984; Windbichler et al. 2000). However, in several cases, novel molecules or combinations have substituted IFN or demoted it to second-line treatment and beyond (Conlon et al. 2018).

Clinical Applications of Interferon Gamma (IFNγ)

IFNγ was initially measured as a capable immunotherapeutic based on the preclinical animal model results and was broadly tested in clinical trials. A phase II trial of IFNγ was delivered to 15 patients by mycosis fungoides intravenously at dose of 2 million units daily for 5 days per week over 4 weeks, after that daily SC administration, exhibited an unbiassed response rate of 60%, with only 1 patient progressing at the median follow-up of 170 days (Sugaya et al. 2014). However, IFNγ showed no effectiveness in oncology patients and only received FDA approvals for nononcologic indications, i.e., chronic granulomatous disease and osteopetrosis. The suggested reason for this lack of efficacy has been initiation of MDSC and slight therapeutic index (Street et al. 2002; Lee and Margolin 2011).

Clinical Applications of Interleukin (IL)-12

Preclinical results remain to demonstrate IL-12's potential as an immunotherapeutic and that is being evaluated in clinical trials. NHS-IL-12, a novel immunokine, is composed of 2 IL-12 molecules merged to a human IgG1 with attraction for exposed single- and double-stranded DNA in parts of tumor necrosis. NHS-IL-12 has a longer half-life, and improved efficacy and toxicity profile in preclinical models (Fallon et al. 2014). This agent is being studied in numerous ongoing clinical trials. Other clinical formulations comprise a new recombinant human protein cell line fabricating IL-12, or transduced effector cells, or virally transduced tumor cells, or cell lysate proposed to stimulate effector T cells locally (Conlon et al. 2018).

Clinical Applications of Interleukin (IL)-2

The Food and Drug Administration (FDA) endorsement of high-dose interleukin-2 (HDIL-2) in the treatment of metastatic renal cell carcinoma (mRCC) and metastatic melanoma (mM) was constructed on data collected from multiple pivotal trials administering IL-2 600,000–720,000 IU/kg with up to 15 bolus infusions every 8 h based on patients' tolerance in 255 patients with mRCC (Rosenberg et al. 1989; Fyfe et al. 1995; Atkins et al. 1999). As the recombinant cytokine was first developed for clinical use, treatment with IL-2 has been assessed in various different dose ranges, schedules, and routes of administration in efforts to maximize efficacy and minimize toxicity (Conlon et al. 2018). IL-2 is nearly universally applied in adoptive T-cell therapy (ACT) with tumor-infiltrating lymphocytes (TILs) gathered from autologous tumor deposits, extended ex vivo, and reinfused with HDIL-2 regimen (Conlon et al.

2018). TIL therapy usually given with IL-2 has been inspected in clinical trials for other solid tumor types with diverse success (Andersen et al. 2015). Low-dose IL-2 therapy provided through continuous intravenous (CIV) infusion over the course of 90 days was related with a substantial growth of $CD56^{+}/CD3^{-}$ NK cells (Caligiuri et al. 1993; Soiffer et al. 1996; Fehniger et al. 2000). Although, such continued low-dose IL-2 therapy was effective in intensifying the NK cells number, these NK cells were not triggered, but required high amounts of IL-2 in vivo to destroy tumor cells. Thus, low-dose IL-2 to increase NK cells was united with transitional pulses of IL-2 to provide an activation of a prolonged NK cell pool (Caligiuri et al. 1993).

Clinical Applications Interleukin (IL)-21

Antitumor activity of IL-21 was reported in several preclinical studies (Skak et al. 2008) that presented growth inhibition in B16 melanoma and MCA205 fibrosarcoma tumors, and amplified survival of tumor-bearing mice (Conlon et al. 2018). IL-21 was combined with cetuximab (Erbitux), an antibody targeting epidermal growth factor receptor, to enhance its ADCC against tumors. In phase I trials, the conjugation of IL-21 with cetuximab against stage IV colorectal cancer stable disease was accomplished in 60% of patients (Steele et al. 2012). However, clinical trial was terminated when IL-21 was revealed to have a role in the growth of chronic inflammatory bowel disease, and in indorsing the inflammation-induced growth of colon cancer (Steele et al. 2012). Eventually, issues with severe hepatic or gastrointestinal (GI) toxicities and lack of reliable clinical activity led to termination of IL-21's clinical formulation by Pharma (Conlon et al. 2018).

Clinical Applications of Interleukin (IL)-7

IL-7 signaling is well acknowledged to occur in either the beginning or preservation of some lymphocyte-derived tumors, such as T-cell acute lymphoblastic leukemia (Barata et al. 2005; Silva et al. 2011). Two phase I dose escalation trials were engaged at the National Cancer Institute (Rosenberg et al. 2006; Sportes et al. 2010). The first in human was a dose-escalation trial that administered subcutaneously (SC) recombinant human IL-7 (rhIL-7) in combination with 2 well-characterized melanoma peptides, i.e., glycoprotein (gp) 100 and melanoma antigen recognized by T-cells 1 (MART-1), typically in patients with malignant melanoma. The therapy was well tolerated, but no objective outcomes were seen. In recent trial, patients with incorrigible nonhematologic malignancies were treated with escalating doses of SC rhIL-7 (3–60 mcg/kg), administrated every other day for 2 weeks, showed no indication of clinical activity (Capitini et al. 2009). Sustained clinical development of IL-7 was halted by the discovery that the original *Escherichia coli* generated material was enormously immunogenic, causing the formation of neutralizing IL-7 antibodies to avert repeated treatment cycles. Thus, IL-7 appears to be intricate in autocrine circuitries to continue the lymphoma cells growth (Cattaruzza et al. 2009).

Clinical Applications of Interleukin (IL)-15

IL-15 was reported with significant therapeutic value against neoplasia in a number of murine models (Evans et al. 1997; Fehniger et al. 2002; Klebanoff et al. 2004; Dubois et al. 2008; Zhang et al. 2009, 2012; Steel et al. 2012; Yu et al. 2012; Waldmann 2015). The antitumor effect in several cases was discovered to be largely dependent on the augmentation of NK cell cytotoxicity and Natural killer group 2 member D (NKG2D)-mediated activation of NK cells. The safety of IL-15 was assessed in *Rhesus macaques* (Mueller et al. 2005; Berger et al. 2009; Lugli et al. 2010; Waldmann et al. 2011). Recombinant human (rh) IL-15 formed in *E. coli* was directed at a dosing schedule of 12 daily IV bolus infusions at doses of 10, 20, and 50 mcg/kg/day to *Rhesus macaques*, with the only biologically meaningfully laboratory deviation being a grade 3/4 transient neutropenia. A 12-day bolus of IV administration of 20 mcg/kg/day of IL-15 to *Rhesus macaques* was related with a four to eightfold upsurge in the amount of circulating NK, central, stem, and effector memory T cells (Lugli et al. 2010; Waldmann et al. 2011). IL-15 is also related with the expression of immunological checkpoints, such as expression of PD-1 on CD8 T cells and the generation of IL-10 and T cell immunoreceptor with Ig and LTim domains (TIGIT) (Yu et al. 2010).

21.5.2.2 Therapeutic Cancer Vaccines

Antigen exhibition is the initial step in the production of immune response by the immune cells. These tumor vaccines worked on the principle is to expose the patients against tumor antigens to provoke an antitumor immune response via the production of tumor specific antibodies and/or T cells. Cancer vaccines induce, produce, and enhance the adaptive antitumor response through amplification of tumor antigen presentation. Thus, cancer vaccines can be broadly grouped into two types: (1) active whole-cell vaccines and (2) specific peptide antigen preparations (Velcheti and Schalper 2016). Similarly, tumor antigens are categorized into two types that are measured as targets for immunotherapy: (1) tumor-specific antigens (TSAs) and (2) tumor-associated antigens (TAAs). TSAs are extremely tumor specific and are expressed only on the surface of tumor cells while TAAs generally grow on the surface of both tumor and non-tumor cells. Under these conditions, the application of TSAs can escape the target autoimmune adverse events, but tumor responses are restricted by the incidence of the target antigen. Although TAAs expressed at higher levels in tumors, they lack strong immune response and higher off-target autoimmune events. These vaccine antigens are generally expressed by APCs, such as macrophages or dendritic cells, to trigger T and B cells, which results in the enhanced antitumor responses (Bitton 2004). The only cancer vaccine, sipuleucel-T, a dendritic cell vaccine, approved by the FDA against metastatic castrate-resistant prostate carcinoma. Herein, dendritic cells are collected from the patients and treated with prostatic acid phosphatase and granulocyte macrophage colony-stimulating factor (GM-CSF) followed by reinfusion into the patient; this treatment results in a 4-month growth in median survival (Gardner et al. 2012). Typically, cancer vaccines are characterized as a type of biologic response modifier; BCG was the first cancer vaccine to be used as therapeutic therapy against bladder carcinoma (Speil and

Rzepka 2011), where it positively altered the expression of tumor antigens following the tumor cells adopt to the co-cultured bacteria. This method induced a high and complex coordinated release of cytokines, such as IL-2, IL-12, IFN-γ, and tumor necrosis factor from the T helper 1 cells while T helper 2 cells released the signal molecules IL-4, IL-5, IL-6, and IL-10. Furthermore, macrophages, epithelial cells, and fibroblasts support IL-8 and T helper 17 cells to secrete IL-17 (Fuge et al. 2015). This huge collection of cytokines then prompt the antitumor activity assisted by NK cells, cytotoxic T lymphocytes, macrophages, and neutrophils. However, clinical benefit induced by the therapeutic vaccines have been uncertain in all three phases' trials. It was suggested because most of the therapeutic cancer vaccines discovered till date have been based on the tumor associated antigens as antigenic material (Sathyanarayanan and Neelapu 2015). Thus, it is expected that vaccination with tumor-associated antigens induces only low to modest affinity T cells because of self-tolerance mechanisms.

Peptide-Based Vaccines

Peptide-based vaccines exhibit an immune response against a single tumor antigen expressed in combination with HLA molecules on the tumor cells surface (Alatrash et al. 2013). These vaccines are predicted to produce less toxicity in normal cells and tissues but lack the specificity in the identification of tumor antigen peptide patient HLA type (Alatrash et al. 2013; Ventola 2017).

21.5.3 Oncolytic Viruses

Oncolytic viruses are the emerging group of cancer therapeutics with a combination of biologic therapy and immunotherapy. In this approach, viruses are genetically altered to remove virulence against normal cells but specially infect and lyse the tumor cells. Cancer cell lysis is one of the multiple mechanisms involved in the oncolytic viruses based cancer therapy; the infected cells are further attacked by immune cells stimulated by a plethora of tumor antigens secreted following lytic destruction of cancer cells (Choi et al. 2016). In 2015, a modified herpes simplex-1 virus (HSV-1) to express GM-CSF, named as "T-VEC (Talimogene laherparepvec)," which further trigger the proliferation of immune cells was approved by the FDA as oncolytic virus for the treatment of advanced melanoma. This oncolytic virus is injected directly at the site of tumor which cannot be removed by surgery. Moreover, other oncolytic viruses are under clinical trials for the treatment of various cancers, including some trials combined with other types of cancer therapies (Oiseth and Aziz 2017).

21.5.4 Cellular/Adoptive Cell Therapies

Cellular immunotherapy, also known as adoptive cell therapy, is a form of therapeutic approach that uses the application of immune system to destroy the cancer. Some

of these approaches include direct collection of patient immune cells followed by their proliferation while other comprise genetical modification of patient immune cells via gene therapy to improve their ability to fight against cancer. Thus, cellular immunotherapy acts by simulating the antitumor activity in patients (tumor and dendritic cell vaccines) or that have intrinsic antitumor activity (autologous and allogeneic lymphocytes). The target of this therapy is to harness the potent immunological weapons to kill the cancer cells (Armstrong et al. 2001). Even with use of highly developed responses, designed immune systems against cancer frequently fail to generate an effect; the immune system becomes blinded to the tumor. The eventual aim of cellular immunotherapy is to get rid of failed immunological responses and provide strong and effective immune system to kill the tumor cells (Armstrong et al. 2001).

21.5.4.1 Dendritic Cell Therapy

Dendritic cells (DCs) were initially recognized by Ralph Steinman as bone marrow (BM) consequent professional APCs, being the only cell of the immune system with ability to activate naive T cells (Steinman and Cohn 1973). Subsequently, DC were reported to "program" the quality of T-cell responses, producing Th1, Th2, Th17 polarization, or in some causes triggering T-cell anergy or T regulatory cell formation. In difference to other APCs like macrophage or B cell, DCs display magnitudes of advanced ability to excite T-cell responses both in antigen specific systems and in polyclonal experiments as in mixed lymphocyte reaction (Banchereau and Steinman 1998). It is known that in peripheral tissues, DCs capture antigens by several complementary mechanisms such as phagocytosis and receptor-mediated endocytosis (Pamer and Cresswell 1998). There is some suggestion that DCs dynamically promote tumor immunogenicity in that patients with DC penetration of tumors generally have a better prognosis (Ayari et al. 2009, 2013; Liska et al. 2012; Hu et al. 2014). The most progressive DC-based therapy is Provenge (sipuleucel-T), approved by the FDA against androgen-resistant prostate cancer. Provenge is produced from monocytes that are encouraged to differentiate to DC by culture in a chimeric protein of GM-CSF prostate-specifc antigen (PSA), and prostatic acid phosphatase (PAP) (Gomella et al. 2014; Sternberg et al. 2014). Herein, dendritic cells are collected from patients' blood and triggered outside the body in the presence of tumor antigens, which may be a single tumor-specific peptide/protein or a tumor cell lysate (a solution of broken-down tumor cells). Following, the activated cells (with optional adjuvants) are infused in the patient body to provoke an enhanced immune response.

21.5.4.2 CAR-T Cell Therapy

Adoptive cell therapy (ACT) is one of the types of immunotherapy which involves the collection and in vitro expansion of tumor-specific T cells, after which it is infused back into the cancer patient. These approaches have also been applied using NK cells as they exhibit rapid and potent immunity against hematological cancers and solid tumor (Guillerey et al. 2016). For example, cell culture of tumor-infiltrating lymphocytes are collected directly from the tumor, isolation and

proliferation of one particular T-cell or engineered T-cells in vitro is conducted to effectively identify and attack tumors; this technique is called as chimeric antigen receptor T-cell (CAR T-cell) therapy as depicted in Fig. 21.6.

The revolutionary CRISPR/Cas9, also known as "CRISPR (clustered regularly interspaced short palindromic repeats)," technique is a simple and efficient method of gene editing compare to previous methods, and was first documented in 2012 (Jinek et al. 2012). The acronym stands for "clustered regularly interspaced short palindromic repeats," which denotes to a process usually used by bacteria and archaea for defense against the attacking nucleic acids of viruses and plasmids. Although Adoptive cellular therapy (ACT) has formed notable results in clinical trials with melanoma and hematologic malignancies, and solid cancers, some deaths have happened in the trial phases secondary to marked cytokine release and cerebral edema. Researchers are revising other ways of adjusting T cells for the cancer treatment. Relapsed and refractory B-cell acute lymphoblastic leukemia in pediatric and young adult patients is the first disease to obtain FDA approval for CAR T-cell therapy, outside of clinical trials (Levine et al. 2017). In contrast to TILs, CAR T cells are accomplishing complete and strong remission in 50–80% of paediatric acute lymphocytic leukaemia, even after all other conduct options have failed (Trapani and Darcy 2017).

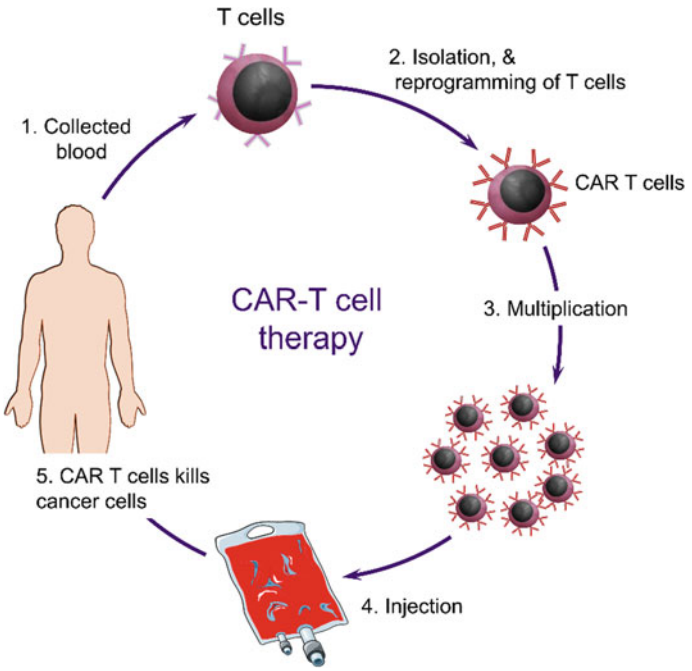


Fig. 21.6 Depiction of CAR T Cell therapy where patients T cells are genetically engineered in the lab to express specific protein called chimeric antigen receptors. The modified CAR T cells are then proliferated in vitro and infused into patient body to destroy the cancer cell

21.5.5 Immune Checkpoint Antagonists/Inhibitors or Stimulatory Agonists

In contrast to therapeutic cancer vaccines that make new antitumor T cells after immunization, immune checkpoint antagonists act by improving the preexisting antitumor T cells function (Fig. 21.7). Immune checkpoint blockade target at natural generation of antitumor T cells in most cancers against tumor antigens (Sathyanarayanan and Neelapu 2015). To ensure that an immune inflammatory response is not continuously activated once foreign or tumor antigens have triggered a response, multiple controls or "checkpoints" are activated. These checkpoints are frequently represented by T-cell receptor binding to the expressed ligands on cells in the surrounding microenvironment, creating immunological synapses to regulate the functions of T cell; these cells further performed the different activities. As noted earlier, initial T-cell activation comprises antigen presentation by the MHC molecules on the antigen presenting cells (APCs) to the equivalent T-cell receptor (TCR) on naive T cells. The communication of the costimulatory T-cell receptor CD28 with the B7 ligand is essentially needed for full stimulation, which is tightly controlled or repressed by inhibitory checkpoint receptor/ligand pairs to evade collateral damage from autoimmunity (Sharma et al. 2017). This type of suppression or tempted dysfunctionality of T cells is also known as "T-cell exhaustion" and is different from senescence or anergy. Although, it is generally reversible physiologic defensive mechanism against autoimmunity, the first reflection of it was made in mice infested with a chronically persistent strain of lymphocytic choriomeningitis virus (Gallimore et al. 1998).

More than 20 checkpoint molecule pairs, both costimulatory and co-inhibitory, have been revealed, such as Lymphocyte-activation gene (LAG)-3/MHCII, T cell immunoreceptor with Ig and ITIM domains (TIGIT)/CD155, and T-cell immunoglobulin mucin-3/Galectin-9 (TIM3/Gal-9), which are variably expressed not only by T cells but also by other cells of lymphoid and myeloid derivation (Catakovic et al. 2017; Tsai and Hsu 2017). Some of these molecules are more commonly known as membrane moieties with important differences: for example, lymphocyte triggered gene-3 (LAG-3) is structurally homologous to CD4 but holds strong-binding affinity with MHC class II antigens compared to CD4. Since, these checkpoint molecules are upregulated in suppressed T cells, commonly used as markers of "T-cell exhaustion." Another approach to improve the function of T cells is to deliver activating signals to costimulatory receptors that are also tempted upon T cell activation. There are a number of known receptors that could serve as targets for agonist antibodies, these include 4-1BB (CD137), OX-40 (CD134), GITR (CD357), CD27, and others (Mellman et al. 2011; Sathyanarayanan and Neelapu 2015). Agents targeting myeloid cells such as macrophages, myeloid derived suppressor cells (MDSC), and dendritic cells can also be used to encourage effector T cell activation (Fig. 21.7).

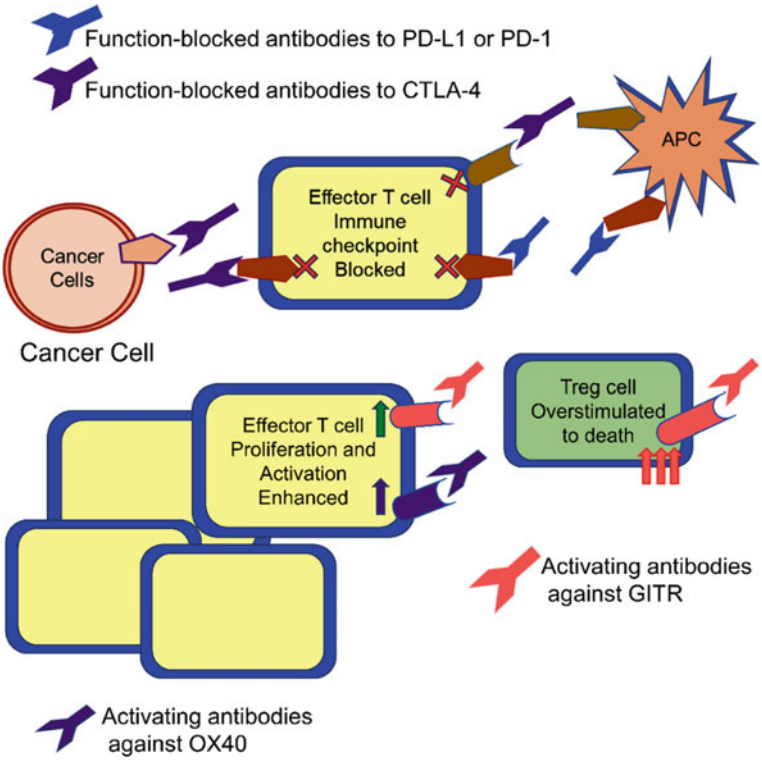


Fig. 21.7 Examples of antagonist and agonist immunotherapies. Some drugs are function-blocking antibodies, such as those targeting the PD-1/PD-L1 interaction, and some are activating antibodies, such as those targeting the OX40/OX40L interaction

21.5.6 Combination of Immunotherapy

Many cancer immunotherapy agents have lately been shown to tempt durable clinical remissions in different type of cancers. But, only a portion of the patients show considerable response with the application of monotherapy. Early consequences from the application of combination immunotherapy strategies against blocking multiple immune resistance mechanisms display that a greater percentage of patients have benefited with combination therapies. For example, combination therapy of nivolumab and ipilimumab triggered responses in over half the patients with reverted melanoma by comparison with 10–30% with monotherapy, although combination therapy was related with higher frequency of immune-related antagonistic events (Wolchok et al. 2013). Emerging results from preclinical studies suggested that combination strategies have significantly improved the responses and probably cured many cancers (Houot et al. 2009; Houot and Levy 2009; Curran et al. 2010; Kohrt et al. 2011, 2014; Marabelle et al. 2013). The application of immunotherapeutic agents in combination to enhance the effector T cell function

with agents that downregulate immunosuppressive elements like Tregs, MDSC, and macrophages in the TME are suggested as complementary and possibly in synergistic therapy (Fig. 21.8).

For instance, certain chemotherapeutic agents like doxorubicin and radiation therapy can cause immunogenic tumor cell death as well as responsible for secretion of tumor neo-antigens in an inflammatory microenvironment and endorse Teff activation. Monoclonal antibodies and targeted therapies for the tumor may also induce a "vaccine-like" effect by inducing immunogenic tumor cell death. Agonistic antibodies directing costimulatory molecules or antagonistic antibodies targeting co-inhibitory molecules on Teffs can be applied as in monotherapy or in combination to enhance the Teff function. Mediators directing regulatory T cells (Tregs) can either prevent the immunosuppressive function or induce depletion of Tregs in the tumor microenvironment (TME). Agents directing myeloid cells, such as myeloid derived suppressor cells (MDSC), macrophages (MF), and dendritic cells (DC), may either twist their polarization to inflammatory state that endorses Teff activation or deplete them from the TEM. Combination therapies comprise these agents are considered to be synergistic and/or complementary, and may significantly advance clinical efficacy and consequence of cancer immunotherapy in the future (Sathyanarayanan and Neelapu 2015). For example, clinical studies merging DC vaccination with radiotherapy, chemotherapy, and/or targeted therapy have been performed (Fig. 21.8) (van Willigen et al. 2018). Moreover, recent studies performed by Kolstad and colleagues presented that combining radiotherapy with intranodal injection of low-dose rituximab, immature dendritic cells, and GM-CSF persuades systemic $CD8^+$ T cell immunity and regression of disseminated follicular lymphoma (Kolstad et al. 2015). Together, these studies established that immunotherapeutic

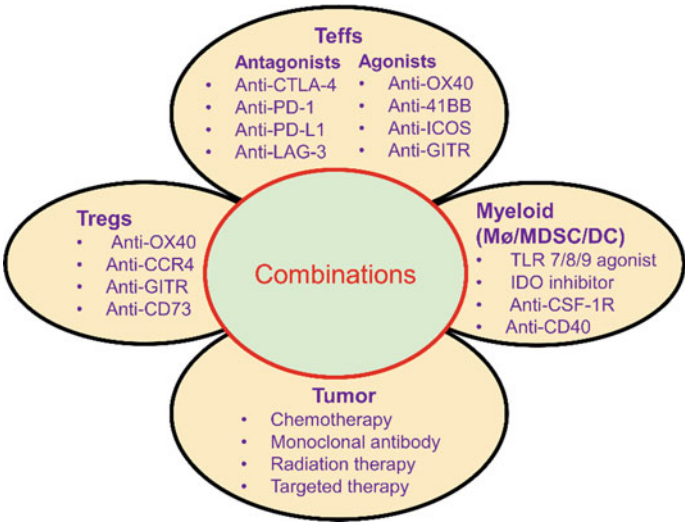


Fig. 21.8 Combination of immunotherapy strategies to enhance function of effector T cells (Teffs)

agents can be combined with conservative chemotherapeutic agents and radiation therapy to improve antitumor immunity and augment clinical outcome.

21.6 Conclusions

With the expansion of the field of cancer immunotherapy, the focus of treatment has shifted from treating the disease site to treating the specific tumor biologic characteristics and its interaction with the intrinsic immunological ability of the patient to war the disease. Since, the immune system has the capacity to remember and the ability to detect and destroy tumor variants as they emerge, immunotherapy will always possess inherent advantages over other therapies that lack these two key attributes. The challenges ahead are to discover why immunotherapy treatments work so dramatically well in some cancers and in some patients while not at all in others, and how tumors which were once sensitive to treatment can acquire resistance. Specifically, to be effective, cancer immunotherapy needs to find ways to manipulate the immune system in the patients who show little or no immune response to their tumors, even to the point where the tumor microenvironment is an "immune desert" with no tumor-infiltrating T cells (Gajewski 2015; Hegde et al. 2016). Different anticancer immunotherapy treatment modalities have the potential to eventually cure and end all forms of cancer. Such great promise and hope is now emerging from a remarkable amount of data that has been accumulated in this field in a very short period of time. Looking ahead to a more promising future for patients with cancer, we must improve our understanding on the mechanism of action of checkpoint inhibitors, CAR-T cell therapy, cytokine-based immunotherapy, etc., and search for more therapeutic biomarkers that are able to predict who would benefit the most from such treatments. The future of immunotherapy will likely involve combinations of immunotherapy, such as vaccines and the immunomodulators, and immunotherapy with new potent inhibitors of the tumor signaling pathways that remit disease in more than one-half of treated subjects. The careful evaluation of immune responses to tumors during the combined application of immunotherapy and other modalities will be critical to defining biomarkers of response and in providing patients with the optimal benefits from rational combinations of more specific immunotherapies and combinations.

References

Adams JL, Smothers J, Srinivasan R, Hoos A (2015) Big opportunities for small molecules in immuno-oncology. Nat Rev Drug Discov 14(9):603–622

Alatrash G, Jakher H, Stafford PD, Mittendorf EA (2013) Cancer immunotherapies, their safety and toxicity. Expert Opin Drug Saf 12(5):631–645

Andersen R, Donia M, Westergaard MC, Pedersen M, Hansen M, Svane IM (2015) Tumor infiltrating lymphocyte therapy for ovarian cancer and renal cell carcinoma. Hum Vaccin Immunother 11(12):2790–2795

Armstrong AC, Eaton D, Ewing JC (2001) Cellular immunotherapy for cancer. BMJ 323(7324): 1289–1293

Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, Abrams J, Sznol M, Parkinson D, Hawkins M, Paradise C, Kunkel L, Rosenberg SA (1999) High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. J Clin Oncol 17(7):2105–2116

Ayari C, LaRue H, Hovington H, Decobert M, Harel F, Bergeron A, Tetu B, Lacombe L, Fradet Y (2009) Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guerin immunotherapy. Eur Urol 55(6):1386–1395

Ayari C, LaRue H, Hovington H, Caron A, Bergeron A, Tetu B, Fradet V, Fradet Y (2013) High level of mature tumor-infiltrating dendritic cells predicts progression to muscle invasion in bladder cancer. Hum Pathol 44(8):1630–1637

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392(6673): 245–252

Barata JT, Cardoso AA, Boussiotis VA (2005) Interleukin-7 in T-cell acute lymphoblastic leukemia: an extrinsic factor supporting leukemogenesis? Leuk Lymphoma 46(4):483–495

Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC, Riddell SR (2009) Safety and immunologic effects of IL-15 administration in nonhuman primates. Blood 114(12):2417–2426

Birbrair A, Zhang T, Wang ZM, Messi ML, Olson JD, Mintz A, Delbono O (2014) Type-2 pericytes participate in normal and tumoral angiogenesis. Am J Physiol Cell Physiol 307(1):C25–C38

Bissell MJ, Radisky D (2001) Putting tumours in context. Nat Rev Cancer 1(1):46–54

Bitton RJ (2004) Cancer vaccines: a critical review on clinical impact. Curr Opin Mol Ther 6(1): 17–26

Buck MD, Sowell RT, Kaech SM, Pearce EL (2017) Metabolic instruction of immunity. Cell 169(4):570–586

Burnet M (1957) Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J 1(5023):841–847

Burnet FM (1970) The concept of immunological surveillance. Prog Exp Tumor Res 13:1–27

Burstein HJ, Krilov L, Aragon-Ching JB, Baxter NN, Chiorean EG, Chow WA, De Groot JF, Devine SM, DuBois SG, El-Deiry WS, Epstein AS, Heymach J, Jones JA, Mayer DK, Miksad RA, Pennell NA, Sabel MS, Schilsky RL, Schuchter LM, Tung N, Winkfield KM, Wirth LJ, Dizon DS (2017) Clinical cancer advances 2017: annual report on progress against cancer from the American Society of Clinical Oncology. J Clin Oncol 35(12):1341–1367

Busch W (1868) Aus der Sitzung der medicinischen Section vom 13 November 1867. Berl Klin Wochenschr 5:137

Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, Schow P, Ross ME, Klumpp TR, Soiffer RJ et al (1993) Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J Clin Invest 91(1):123–132

Capitini CM, Chisti AA, Mackall CL (2009) Modulating T-cell homeostasis with IL-7: preclinical and clinical studies. J Intern Med 266(2):141–153

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A 72(9):3666–3670

Catakovic K, Klieser E, Neureiter D, Geisberger R (2017) T cell exhaustion: from pathophysiological basics to tumor immunotherapy. Cell Commun Signal 15(1):1

Cattaruzza L, Gloghini A, Olivo K, Di Francia R, Lorenzon D, De Filippi R, Carbone A, Colombatti A, Pinto A, Aldinucci D (2009) Functional coexpression of Interleukin (IL)-7 and its receptor (IL-7R) on Hodgkin and Reed-Sternberg cells: involvement of IL-7 in tumor cell growth and microenvironmental interactions of Hodgkin's lymphoma. Int J Cancer 125(5): 1092–1101

Challis GB, Stam HJ (1990) The spontaneous regression of cancer. A review of cases from 1900 to 1987. Acta Oncol 29(5):545–550

Chambers AF, Werb Z (2015) Invasion and metastasis—recent advances and future challenges. J Mol Med (Berl) 93(4):361–368

Champiat S, Lambotte O, Barreau E, Belkhir R, Berdelou A, Carbonnel F, Cauquil C, Chanson P, Collins M, Durrbach A, Ederhy S, Feuillet S, Francois H, Lazarovici J, Le Pavec J, De Martin E, Mateus C, Michot JM, Samuel D, Soria JC, Robert C, Eggermont A, Marabelle A (2016) Management of immune checkpoint blockade dysimmune toxicities: a collaborative position paper. Ann Oncol 27(4):559–574

Chen DS, Mellman I (2017) Elements of cancer immunity and the cancer-immune set point. Nature 541(7637):321–330

Cheson BD, Leonard JP (2008) Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma. N Engl J Med 359(6):613–626

Chida K, Nakanishi K, Shomura H, Homma S, Hattori A, Kazui K, Taketomi A (2017) Spontaneous regression of transverse colon cancer: a case report. Surg Case Rep 3(1):65

Chien YH, Jores R, Crowley MP (1996) Recognition by gamma/delta T cells. Annu Rev Immunol 14:511–532

Choi AH, O'Leary MP, Fong Y, Chen NG (2016) From benchtop to bedside: a review of oncolytic virotherapy. Biomedicines 4(3):18

Coley WB (1910) The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med 3(Surg Sect):1–48

Coley WB (1991) The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. Clin Orthop Relat Res (262):3–11

Conlon KC, Miljkovic MD, Waldmann TA (2018) Cytokines in the treatment of cancer. J Interf Cytokine Res 39(1):6–21

Cooper GM (1992) Elements of human cancer. Jones & Bartlett Learning

Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420(6917):860–867

Curran MA, Montalvo W, Yagita H, Allison JP (2010) PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A 107(9):4275–4280

Dinarello CA (2007) Historical insights into cytokines. Eur J Immunol 37(Suppl 1):S34–S45

Dominguez-Villar M, Gautron AS, de Marcken M, Keller MJ, Hafler DA (2015) TLR7 induces anergy in human CD4(+) T cells. Nat Immunol 16(1):118–128

Dubois S, Patel HJ, Zhang M, Waldmann TA, Muller JR (2008) Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. J Immunol 180(4):2099–2106

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3(11):991–998

Ehrlich P (1906) Collected studies on immunity. Wiley

Evans R, Fuller JA, Christianson G, Krupke DM, Troutt AB (1997) IL-15 mediates anti-tumor effects after cyclophosphamide injection of tumor-bearing mice and enhances adoptive immunotherapy: the potential role of NK cell subpopulations. Cell Immunol 179(1):66–73

Fallon J, Tighe R, Kradjian G, Guzman W, Bernhardt A, Neuteboom B, Lan Y, Sabzevari H, Schlom J, Greiner JW (2014) The immunocytokine NHS-IL12 as a potential cancer therapeutic. Oncotarget 5(7):1869–1884

Fehleisen F (1882) Ueber die Züchtung der Erysipelkokken auf künstlichem Nährboden und ihre Übertragbarkeit auf den Menschen. Dtsch Med Wochenschr 8(31):553–554

Fehniger TA, Bluman EM, Porter MM, Mrozek E, Cooper MA, VanDeusen JB, Frankel SR, Stock W, Caligiuri MA (2000) Potential mechanisms of human natural killer cell expansion in vivo during low-dose IL-2 therapy. J Clin Invest 106(1):117–124

Fehniger TA, Cooper MA, Caligiuri MA (2002) Interleukin-2 and interleukin-15: immunotherapy for cancer. Cytokine Growth Factor Rev 13(2):169–183

Fuge O, Vasdev N, Allchorne P, Green JS (2015) Immunotherapy for bladder cancer. Res Rep Urol 7:65–79

Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, Louie AC (1995) Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. J Clin Oncol 13(3):688–696

Gajewski TF (2015) The next hurdle in cancer immunotherapy: overcoming the non-T-cell-inflamed tumor microenvironment. Semin Oncol 42(4):663–671

Galli F, Aguilera JV, Palermo B, Markovic SN, Nisticò P, Signore A (2020) Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy. J Exp Clin Cancer Res 39(1):89

Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, Hengartner H, Zinkernagel R (1998) Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. J Exp Med 187(9):1383–1393

Gardner TA, Elzey BD, Hahn NM (2012) Sipuleucel-T (Provenge) autologous vaccine approved for treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer. Hum Vaccin Immunother 8(4):534–539

Garrido F, Aptsiauri N, Doorduijn EM, Garcia Lora AM, van Hall T (2016) The urgent need to recover MHC class I in cancers for effective immunotherapy. Curr Opin Immunol 39:44–51

Gery I, Waksman BH (1972) Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). J Exp Med 136(1):143–155

Gomella LG, Gelpi-Hammerschmidt F, Kundavram C (2014) Practical guide to immunotherapy in castration resistant prostate cancer: the use of sipuleucel-T immunotherapy. Can J Urol 21(2 Suppl 1):48–56

Gonzalez H, Hagerling C, Werb Z (2018a) Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes Dev 32(19–20):1267–1284

Gonzalez H, Robles I, Werb Z (2018b) Innate and acquired immune surveillance in the postdissemination phase of metastasis. FEBS J 285(4):654–664

Guillerey C, Huntington ND, Smyth MJ (2016) Targeting natural killer cells in cancer immunotherapy. Nat Immunol 17(9):1025–1036

Gutterman JU, Blumenschein GR, Alexanian R, Yap HY, Buzdar AU, Cabanillas F, Hortobagyi GN, Hersh EM, Rasmussen SL, Harmon M, Kramer M, Pestka S (1980) Leukocyte interferon-induced tumor regression in human metastatic breast cancer, multiple myeloma, and malignant lymphoma. Ann Intern Med 93(3):399–406

Haanen J, Carbonnel F, Robert C, Kerr KM, Peters S, Larkin J, Jordan K, ESMO Guidelines Committee (2017) Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 28(Suppl 4):iv119–iv142. clinicalguidelines@esmo.org

Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21(3):309–322

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674

Hegde PS, Karanikas V, Evers S (2016) The where, the when, and the how of immune monitoring for cancer immunotherapies in the era of checkpoint inhibition. Clin Cancer Res 22(8): 1865–1874

Hosseini H, Obradovic MMS, Hoffmann M, Harper KL, Sosa MS, Werner-Klein M, Nanduri LK, Werno C, Ehrl C, Maneck M, Patwary N, Haunschild G, Guzvic M, Reimelt C, Grauvogl M, Eichner N, Weber F, Hartkopf AD, Taran FA, Brucker SY, Fehm T, Rack B, Buchholz S, Spang R, Meister G, Aguirre-Ghiso JA, Klein CA (2016) Early dissemination seeds metastasis in breast cancer. Nature 540(7634):552–558

Houot R, Levy R (2009) T-cell modulation combined with intratumoral CpG cures lymphoma in a mouse model without the need for chemotherapy. Blood 113(15):3546–3552

Houot R, Goldstein MJ, Kohrt HE, Myklebust JH, Alizadeh AA, Lin JT, Irish JM, Torchia JA, Kolstad A, Chen L, Levy R (2009) Therapeutic effect of CD137 immunomodulation in lymphoma and its enhancement by Treg depletion. Blood 114(16):3431–3438

Hu M, Li K, Maskey N, Xu Z, Peng C, Wang B, Li Y, Yang G (2014) Decreased intratumoral Foxp3 Tregs and increased dendritic cell density by neoadjuvant chemotherapy associated with favorable prognosis in advanced gastric cancer. Int J Clin Exp Pathol 7(8):4685–4694

Iwai Y, Hamanishi J, Chamoto K, Honjo T (2017) Cancer immunotherapies targeting the PD-1 signaling pathway. J Biomed Sci 24(1):26

Janeway CA Jr, Travers P, Walport M, Shlomchik MJ (2001) B-cell activation by armed helper T cells. In: Immunobiology: the immune system in health and disease, 5th edn. Garland Science

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096): 816–821

Kirkwood JM, Ernstoff MS (1984) Interferons in the treatment of human cancer. J Clin Oncol 2(4): 336–352

Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP (2004) IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. Proc Natl Acad Sci U S A 101(7):1969–1974

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497

Kohrt HE, Houot R, Goldstein MJ, Weiskopf K, Alizadeh AA, Brody J, Muller A, Pachynski R, Czerwinski D, Coutre S, Chao MP, Chen L, Tedder TF, Levy R (2011) CD137 stimulation enhances the antilymphoma activity of anti-CD20 antibodies. Blood 117(8):2423–2432

Kohrt HE, Thielens A, Marabelle A, Sagiv-Barfi I, Sola C, Chanuc F, Fuseri N, Bonnafous C, Czerwinski D, Rajapaksa A, Waller E, Ugolini S, Vivier E, Romagne F, Levy R, Blery M, Andre P (2014) Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. Blood 123(5):678–686

Kolstad A, Kumari S, Walczak M, Madsbu U, Hagtvedt T, Bogsrud TV, Kvalheim G, Holte H, Aurlien E, Delabie J, Tierens A, Olweus J (2015) Sequential intranodal immunotherapy induces antitumor immunity and correlated regression of disseminated follicular lymphoma. Blood 125(1):82–89

Koretzky GA (2010) Multiple roles of CD4 and CD8 in T cell activation. J Immunol 185(5): 2643–2644

Kraeber-Bodere F, Bodet-Milin C, Rousseau C, Eugene T, Pallardy A, Frampas E, Carlier T, Ferrer L, Gaschet J, Davodeau F, Gestin JF, Faivre-Chauvet A, Barbet J, Cherel M (2014) Radioimmunoconjugates for the treatment of cancer. Semin Oncol 41(5):613–622

Kroschinsky F, Stolzel F, von Bonin S, Beutel G, Kochanek M, Kiehl M, Schellongowski P, Intensive Care in Hematological and Oncological Patients (iCHOP) Collaborative Group (2017) New drugs, new toxicities: severe side effects of modern targeted and immunotherapy of cancer and their management. Crit Care 21(1):89

Kucerova P, Cervinkova M (2016) Spontaneous regression of tumour and the role of microbial infection—possibilities for cancer treatment. Anti-Cancer Drugs 27(4):269–277

Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30(1):16–34

Lambert AW, Pattabiraman DR, Weinberg RA (2017) Emerging biological principles of metastasis. Cell 168(4):670–691

Larson C, Oronsky B, Scicinski J, Fanger GR, Stirn M, Oronsky A, Reid TR (2015) Going viral: a review of replication-selective oncolytic adenoviruses. Oncotarget 6(24):19976–19989

Leach DR, Krummel MF, Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. Science 271(5256):1734–1736

Lee S, Margolin K (2011) Cytokines in cancer immunotherapy. Cancers (Basel) 3(4):3856–3893

Lei X, Lei Y, Li J-K, Du W-X, Li R-G, Yang J, Li J, Li F, Tan H-B (2020) Immune cells within the tumor microenvironment: biological functions and roles in cancer immunotherapy. Cancer Lett 470:126–133

Levine BL, Miskin J, Wonnacott K, Keir C (2017) Global manufacturing of CAR T cell therapy. Mol Ther Methods Clin Dev 4:92–101

Lin W-W, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117(5):1175–1183

Liska V, Vycital O, Daum O, Novak P, Treska V, Bruha J, Pitule P, Holubec L (2012) Infiltration of colorectal carcinoma by S100+ dendritic cells and CD57+ lymphocytes as independent prognostic factors after radical surgical treatment. Anticancer Res 32(5):2129–2132

Luckheeram RV, Zhou R, Verma AD, Xia B (2012) CD4(+)T cells: differentiation and functions. Clin Dev Immunol 2012:925135

Lugli E, Goldman CK, Perera LP, Smedley J, Pung R, Yovandich JL, Creekmore SP, Waldmann TA, Roederer M (2010) Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. Blood 116(17):3238–3248

Marabelle A, Kohrt H, Sagiv-Barfi I, Ajami B, Axtell RC, Zhou G, Rajapaksa R, Green MR, Torchia J, Brody J, Luong R, Rosenblum MD, Steinman L, Levitsky HI, Tse V, Levy R (2013) Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. J Clin Invest 123(6):2447–2463

Martini F (2006) Fundamentals of anatomy and physiology, 7th edn. Pearson Benjamin Cummings, San Francisco

Mbongue JC, Nicholas DA, Torrez TW, Kim NS, Firek AF, Langridge WH (2015) The role of indoleamine 2, 3-dioxygenase in immune suppression and autoimmunity. Vaccines (Basel) 3(3):703–729

McAllister SS, Weinberg RA (2014) The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. Nat Cell Biol 16(8):717–727

McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. J Clin Oncol 16(8): 2825–2833

Mellman I, Coukos G, Dranoff G (2011) Cancer immunotherapy comes of age. Nature 480(7378): 480–489

Michot JM, Bigenwald C, Champiat S, Collins M, Carbonnel F, Postel-Vinay S, Berdelou A, Varga A, Bahleda R, Hollebecque A, Massard C, Fuerea A, Ribrag V, Gazzah A, Armand JP, Amellal N, Angevin E, Noel N, Boutros C, Mateus C, Robert C, Soria JC, Marabelle A, Lambotte O (2016) Immune-related adverse events with immune checkpoint blockade: a comprehensive review. Eur J Cancer 54:139–148

Mino-Kenudson M (2016) Programmed cell death ligand-1 (PD-L1) expression by immunohistochemistry: could it be predictive and/or prognostic in non-small cell lung cancer? Cancer Biol Med 13(2):157–170

Mitchell MS (1976) An introduction to tumor immunology and immunotherapy. Gynecol Oncol 4(1):1–12

Mitchell MS, Mokyr MB, Aspnes GT, McIntosh S (1973) Cytophilic antibodies in man. Ann Intern Med 79(3):333–339

Mitchell MS, Mokyr MB, Davis JM (1977) Effect of chemotherapy and immunotherapy on tumor-specific immunity in melanoma. J Clin Invest 59(6):1017–1026

Morales A, Eidinger D, Bruce AW (1976) Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J Urol 116(2):180–183

Mueller YM, Petrovas C, Bojczuk PM, Dimitriou ID, Beer B, Silvera P, Villinger F, Cairns JS, Gracely EJ, Lewis MG, Katsikis PD (2005) Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. J Virol 79(8): 4877–4885

Murphy K, Travers P, Walport M (2008a) Principles of innate and adaptive immunity. In: Janeway's immunobiology, pp 1–38

Murphy K, Travers P, Walport M (2008b) Structural variation in immunoglobulin constant regions. In: Janeway's immunobiology, pp 143–213

Naidoo J, Wang X, Woo KM, Iyriboz T, Halpenny D, Cunningham J, Chaft JE, Segal NH, Callahan MK, Lesokhin AM, Rosenberg J, Voss MH, Rudin CM, Rizvi H, Hou X, Rodriguez K, Albano M, Gordon RA, Leduc C, Rekhtman N, Harris B, Menzies AM, Guminski AD, Carlino

MS, Kong BY, Wolchok JD, Postow MA, Long GV, Hellmann MD (2017) Pneumonitis in patients treated with anti-programmed death-1/programmed death ligand 1 therapy. J Clin Oncol 35(7):709–717

Oelschlaeger TA (2010) Bacteria as tumor therapeutics? Bioeng Bugs 1(2):146–147

Oiseth SJ, Aziz MS (2017) Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead. J Cancer Metastasis Treat 3:250–261

Old LJ, Clarke DA, Benacerraf B (1959) Effect of Bacillus Calmette-Guerin infection on transplanted tumours in the mouse. Nature 184(Suppl 5):291–292

Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, Jegasothy B, Wood G, Gordon M, Heald P, Oseroff A, Pinter-Brown L, Bowen G, Kuzel T, Fivenson D, Foss F, Glode M, Molina A, Knobler E, Stewart S, Cooper K, Stevens S, Craig F, Reuben J, Bacha P, Nichols J (2001) Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol 19(2):376–388

Pamer E, Cresswell P (1998) Mechanisms of MHC class I—restricted antigen processing. Annu Rev Immunol 16:323–358

Parish CR (2003) Cancer immunotherapy: the past, the present and the future. Immunol Cell Biol 81(2):106–113

Pickup MW, Mouw JK, Weaver VM (2014) The extracellular matrix modulates the hallmarks of cancer. EMBO Rep 15(12):1243–1253

Podojil JR, Miller SD (2009) Molecular mechanisms of T-cell receptor and costimulatory molecule ligation/blockade in autoimmune disease therapy. Immunol Rev 229(1):337–355

Pugh MB (2000) Stedman's medical dictionary. Lippincott Williams & Wilkins

Ritprajak P, Azuma M (2015) Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma. Oral Oncol 51(3):221–228

Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, Daud A, Carlino MS, McNeil C, Lotem M, Larkin J, Lorigan P, Neyns B, Blank CU, Hamid O, Mateus C, Shapira-Frommer R, Kosh M, Zhou H, Ibrahim N, Ebbinghaus S, Ribas A, KEYNOTE-006 Investigators (2015) Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 372(26):2521–2532

Rosenberg SA, Lotze MT, Yang JC, Aebersold PM, Linehan WM, Seipp CA, White DE (1989) Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. Ann Surg 210(4):474–484; discussion 484–475

Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, Stetler-Stevenson M, Morton KE, Mavroukakis SA, Morre M, Buffet R, Mackall CL, Gress RE (2006) IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. J Immunother 29(3):313–319

Sathyanarayanan V, Neelapu SS (2015) Cancer immunotherapy: strategies for personalization and combinatorial approaches. Mol Oncol 9(10):2043–2053

Schroeder HW Jr, Cavacini L (2010) Structure and function of immunoglobulins. J Allergy Clin Immunol 125(2):S41–S52

Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A (2017) Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 168(4):707–723

Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. CA Cancer J Clin 66(1):7–30

Silva A, Laranjeira AB, Martins LR, Cardoso BA, Demengeot J, Yunes JA, Seddon B, Barata JT (2011) IL-7 contributes to the progression of human T-cell acute lymphoblastic leukemias. Cancer Res 71(14):4780–4789

Skak K, Kragh M, Hausman D, Smyth MJ, Sivakumar PV (2008) Interleukin 21: combination strategies for cancer therapy. Nat Rev Drug Discov 7(3):231–240

Soiffer RJ, Murray C, Shapiro C, Collins H, Chartier S, Lazo S, Ritz J (1996) Expansion and manipulation of natural killer cells in patients with metastatic cancer by low-dose continuous infusion and intermittent bolus administration of interleukin 2. Clin Cancer Res 2(3):493–499

Speil C, Rzepka R (2011) Vaccines and vaccine adjuvants as biological response modifiers. Infect Dis Clin N Am 25(4):755–772

Sportes C, Babb RR, Krumlauf MC, Hakim FT, Steinberg SM, Chow CK, Brown MR, Fleisher TA, Noel P, Maric I, Stetler-Stevenson M, Engel J, Buffet R, Morre M, Amato RJ, Pecora A, Mackall CL, Gress RE (2010) Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. Clin Cancer Res 16(2):727–735

Steel JC, Waldmann TA, Morris JC (2012) Interleukin-15 biology and its therapeutic implications in cancer. Trends Pharmacol Sci 33(1):35–41

Steele N, Anthony A, Saunders M, Esmarck B, Ehrnrooth E, Kristjansen PE, Nihlen A, Hansen LT, Cassidy J (2012) A phase 1 trial of recombinant human IL-21 in combination with cetuximab in patients with metastatic colorectal cancer. Br J Cancer 106(5):793–798

Steinman RM, Cohn ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137(5):1142–1162

Sternberg CN, Petrylak DP, Madan RA, Parker C (2014) Progress in the treatment of advanced prostate cancer. Am Soc Clin Oncol Educ Book 34(1):117–131

Street SE, Trapani JA, MacGregor D, Smyth MJ (2002) Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med 196(1):129–134

Sugaya M, Tokura Y, Hamada T, Tsuboi R, Moroi Y, Nakahara T, Amano M, Ishida S, Watanabe D, Tani M, Ihn H, Aoi J, Iwatsuki K (2014) Phase II study of i.v. interferon-gamma in Japanese patients with mycosis fungoides. J Dermatol 41(1):50–56

Sun JC, Lanier LL (2009) Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? Eur J Immunol 39(8):2059–2064

Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, Panoskaltsis N (2006) Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med 355(10):1018–1028

Teng MW, Galon J, Fridman WH, Smyth MJ (2015) From mice to humans: developments in cancer immunoediting. J Clin Invest 125(9):3338–3346

Terabe M, Berzofsky JA (2008) The role of NKT cells in tumor immunity. Adv Cancer Res 101: 277–348

Thomas L, Lawrence H (1959) Cellular and humoral aspects of the hypersensitive states. Hoeber-Harper, New York, pp 529–532

Trapani J, Darcy P (2017) Immunotherapy of cancer. Aust Fam Physician 46:194–199

Tsai HF, Hsu PN (2017) Cancer immunotherapy by targeting immune checkpoints: mechanism of T cell dysfunction in cancer immunity and new therapeutic targets. J Biomed Sci 24(1):35

Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J, Rougier P (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 360(14):1408–1417

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647

van Willigen WW, Bloemendal M, Gerritsen WR, Schreibelt G, de Vries IJM, Bol KF (2018) Dendritic cell cancer therapy: vaccinating the right patient at the right time. Front Immunol 9: 2265

Velcheti V, Schalper K (2016) Basic overview of current immunotherapy approaches in cancer. Am Soc Clin Oncol Educ Book 35:298–308

Ventola CL (2017) Cancer immunotherapy, part 1: current strategies and agents. P T 42(6):375–383

Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20(3):719–726

Wagner TC, Velichko S, Chesney SK, Biroc S, Harde D, Vogel D, Croze E (2004) Interferon receptor expression regulates the antiproliferative effects of interferons on cancer cells and solid tumors. Int J Cancer 111(1):32–42

Waldmann TA (2015) The shared and contrasting roles of IL2 and IL15 in the life and death of normal and neoplastic lymphocytes: implications for cancer therapy. Cancer Immunol Res 3(3): 219–227

Waldmann TA, Lugli E, Roederer M, Perera LP, Smedley JV, Macallister RP, Goldman CK, Bryant BR, Decker JM, Fleisher TA, Lane HC, Sneller MC, Kurlander RJ, Kleiner DE, Pletcher JM, Figg WD, Yovandich JL, Creekmore SP (2011) Safety (toxicity), pharmacokinetics, immunogenicity, and impact on elements of the normal immune system of recombinant human IL-15 in rhesus macaques. Blood 117(18):4787–4795

Weiner LM, Surana R, Wang S (2010) Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 10(5):317–327

Wills-Karp M (2007) Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. Proc Am Thorac Soc 4(3):247–251

Windbichler GH, Hausmaninger H, Stummvoll W, Graf AH, Kainz C, Lahodny J, Denison U, Muller-Holzner E, Marth C (2000) Interferon-gamma in the first-line therapy of ovarian cancer: a randomized phase III trial. Br J Cancer 82(6):1138–1144

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369(2):122–133

Younes A, Bartlett NL, Leonard JP, Kennedy DA, Lynch CM, Sievers EL, Forero-Torres A (2010) Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. N Engl J Med 363(19): 1812–1821

Yu P, Steel JC, Zhang M, Morris JC, Waldmann TA (2010) Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model. Clin Cancer Res 16(24):6019–6028

Yu P, Steel JC, Zhang M, Morris JC, Waitz R, Fasso M, Allison JP, Waldmann TA (2012) Simultaneous inhibition of two regulatory T-cell subsets enhanced Interleukin-15 efficacy in a prostate tumor model. Proc Natl Acad Sci U S A 109(16):6187–6192

Zhang M, Yao Z, Dubois S, Ju W, Müller JR, Waldmann TA (2009) Interleukin-15 combined with an anti-CD40 antibody provides enhanced therapeutic efficacy for murine models of colon cancer. Proc Natl Acad Sci 106(18):7513–7518

Zhang M, Ju W, Yao Z, Yu P, Wei B-R, Simpson RM, Waitz R, Fassò M, Allison JP, Waldmann TA (2012) Augmented IL-15Rα expression by CD40 activation is critical in synergistic CD8 T cell-mediated antitumor activity of anti-CD40 antibody with IL-15 in TRAMP-C2 tumors in mice. J Immunol 188(12):6156–6164

Zolot RS, Basu S, Million RP (2013) Antibody-drug conjugates. Nat Rev Drug Discov 12(4): 259–260

Thrombotic Complications in Women: Risks and Prevention

22

Swati Srivastava, Iti Garg, Lilly Ganju, Rajeev Varshney, and Bhuvnesh Kumar

Abstract

Two main clinical manifestations of venous thrombo-embolism (VTE) include deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT refers to a blood clot that starts in vein, usually in the deep veins of the legs or pelvis area. This blood clot (thrombus) may dislodge from its site of generation and travel through blood stream into lungs, causing PE, a potentially fatal condition. While incidence rate of VTE vary among different age groups and population, various reports are available on its differential occurrence in men and women, with contrasting data. Although any individual can succumb to VTE due to various inherited and acquired risk factors, the majority of the published data indicates that women are more prone to first incidence of venous thrombosis during different stages of life (from puberty till menopause), while men are more prone to recurrent VTE. The prevention and treatment of VTE thus poses distinct gender-specific challenges. It is extremely important for women to know the stages of life when they are more prone to develop DVT/VTE. Subsequently, women also need to have information about the risks associated with treatments using oral contraceptives, ovarian stimulation, pregnancy, etc. Thus the aim of this review is to (1) assess the incidence and risk factors of VTE in women and (2) to summarize the current guidelines and recommendation of VTE management. Early diagnosis of signs and symptoms of DVT/VTE and use of systematic anticoagulation therapy can prevent progression of thrombus and subsequent PE.

S. Srivastava · I. Garg · L. Ganju · R. Varshney · B. Kumar (✉)
Defence Institute of Physiology and Allied Sciences (DIPAS), Defence Research and Development Organization (DRDO), Delhi, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_22

Keywords

Deep vein thrombosis · Venous thrombo-embolism · Ovarian stimulation · Oral contraceptives · Pregnancy · Hormone replacement therapy

Abbreviations

COCs	Combined oral contraceptives
DVT	Deep vein thrombosis
HRT	Hormone replacement therapy
OHSS	Ovarian hyperstimulation syndrome
PE	Pulmonary embolism
UFH	Unfractioned heparin
VKAs	Vitamin K-antagonists
VTE	Venous thrombo-embolism

22.1 Introduction

Formation of "thrombus" is a complex phenomenon that occurs as a result of blood clot formation, which is due to an imbalance of procoagulant, anticoagulant, and fibrinolytic factors. Arterial thrombosis is almost invariably superimposed on vessel walls, i.e., atherosclerosis. Its symptoms are acute, leading to blocking of vital blood flow to an organ. In contrast to this, development of clot is relatively sudden in case of venous thrombosis (Rosendaal 2005). In general, venous thrombo-embolism (VTE) is a complex multi-factorial disease, which involves interaction of various environmental, acquired, and genetic risk factors. It is clinically represented by mainly deep vein thrombosis (DVT) and pulmonary embolism (PE). Venous thrombo-embolism is a major health concern worldwide and is the third largest leading cause of deaths in the Western world. Annual incidence of VTE ranges from 1 per 1000 to 3 per 1000 in normal populations (Nordström et al. 1992). In Western countries, incidence rate of VTE ranges from 114 to 184 cases per 100,000 individuals (Delluc et al. 2016; Johansson et al. 2014; Alotaibi et al. 2016), and the older population is at increased risk due to acquired risk factors (Heit et al. 2016). However, there are several reports that demonstrate that the incidences of VTE are significantly lower in Asian population compared to Western population (Molina et al. 2009; White et al. 2005). There could be several possible explanations for this: firstly there is a limited availability of epidemiological data in Asia; secondly underdiagnosis of VTE in Asian patients as a result of low awareness and symptomatic nature of VTE (Zakai and McClure 2011); or finally it could also be attributed to the low prevalence of risk factors, such as obesity and mutations, in prothrombin or factor V Leiden genes (Barnes et al. 2008; Jun et al. 2006).

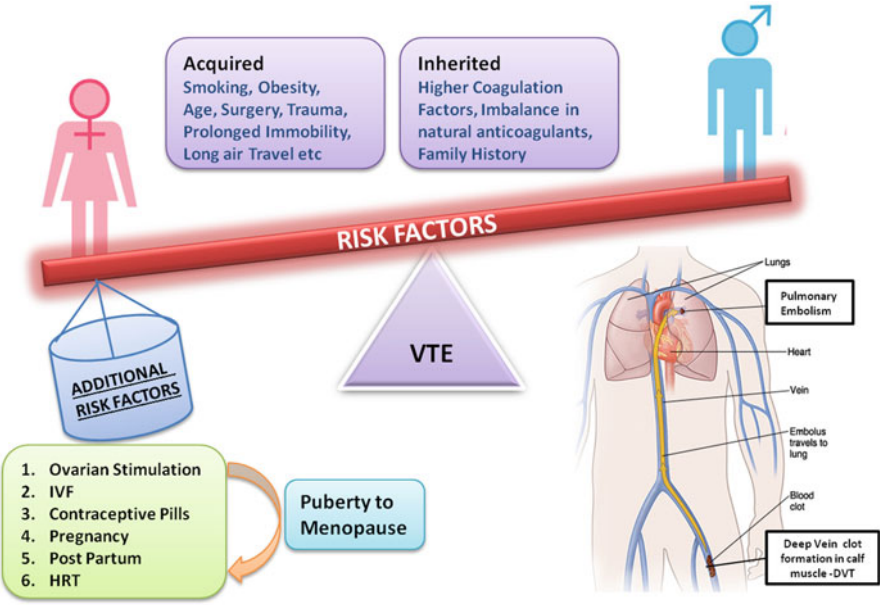


Fig. 22.1 The figure represents the common risk factors (acquired and inherited) of VTE in men and women along with additional risk factors that women possess from the age of puberty till menopause

Both acquired and inherited factors play essential roles in development of VTE (Fig. 22.1). Acquired risk factors convincingly demonstrated for VTE include increasing age, prolonged immobility, malignancy, major surgery, multiple trauma, prior VTE, prolonged air travel, etc. (Rosendaal and Reitsma 2009). Amongst the inherited risk factors, deficiencies of some natural coagulation inhibitors including antithrombin (AT), protein C (PC), and its cofactor protein S (PS), insufficiency of anticoagulant pathways such as tissue factor pathway inhibitor (TFPI), thrombomodulin and endothelial protein C receptor (EPCR), elevated level of factor VIII, mutation in the factor V Leiden (FVL), mutation in the 3′-untranslated part of the prothrombin (Factor II) gene, etc., are included (De Stefano et al. 1996; Ota et al. 2011; Koster et al. 1995).

DVT most commonly starts in the leg, although it rarely also occurs in other veins such as upper extremities, liver, cerebral sinus, retina, and mesenteric. A DVT can be asymptomatic, but in most cases the affected extremity is painful, swollen, red, warm, and the superficial veins may be engorged. It is most commonly diagnosed by blood test called D-dimer test and doppler ultrasound of affected veins. The formation of thrombus is controlled by the coagulation system, which is an extremely essential homeostatic mechanism that prevents excessive bleeding from injuries. The body has to maintain a balanced coagulation system to avoid excessive bleeding as well as thrombus formation. The significance of the coagulation system for the development of thrombosis was observed as early as in 1874 by Virchow in the

Virchow's triad (Blann and Lip 2001). Virchow suggested that thrombosis is either caused by changes in the composition of the blood affecting the coagulation system, in the vessel wall or by changes in blood flow.

The incidence of first venous thrombosis has been assessed in many large cohort studies for men and women separately (Silverstein et al. 1998; Anderson Jr et al. 1991; White et al. 2005; Oger 2000) and the results have been contrasting! Some studies have shown slightly higher risk of venous thrombosis in men compared to women, with a ratio of 1.2:1 (Silverstein et al. 1998; Anderson Jr et al. 1991) whereas others show higher incidence of VTE in women (upto 1.3 folds) compared to men (White et al. 2005; Oger 2000; Naess et al. 2007; Spencer et al. 2009). Women were more likely to have a distal DVT (located below the knee in the calf veins), while men had a higher proportion of proximal DVT (located in the popliteal, femoral, or iliac veins) (Trinchero et al. 2018). Roach and co-workers (2014) performed Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis (MEGA) study and found that overall incidences of first venous thrombosis in men is 2.1-fold (95% confidence interval, 1.9–2.4) higher than in women without reproductive risk factors. However, the risk in women increases twice as compared to men in presence of female reproductive risk factors (Roach et al. 2014a, b). Several studies have independently established that risk of recurrent VTE is higher in men compared to women (Kyrle et al. 2004; Linnemann et al. 2008; Christiansen et al. 2010). Another report of meta-analysis of seven prospective studies calculated 3-year incidence of VTE recurrence revealing 19.7% recurrence rate (16.5–23.4%) in men compared to 9.1% (7.3–11.3%) in women, and it remained higher in men (HR 2.2, CI 1.7–2.8) after adjustment for women with hormone-associated initial VTE (Douketis et al. 2011). However, it is interesting to note that Laczkovics and coworkers reported a higher than expected risk of recurrent VTE in a large series of young women (<45 years age), which is otherwise believed to be at a lower risk (Laczkovics et al. 2007). Coutinho and co-workers identified important differences between women and men in presentation, course, and risk factors of cerebral venous and sinus thrombosis and highlighted that women with a gender-specific risk factor have a much better prognosis than other patients (Coutinho et al. 2009).

Urban women are extremely busy in balancing professional and personal life and often tend to ignore the signs of ill health our body conveys to us. The present article emphasizes on recent studies and their key findings on risk of DVT in women in various stages of life. We intend to draw the focus of women on understanding and assessing the increased risk of VTE, for its early diagnosis and timely treatment.

22.2 Pathophysiology of Venous Thrombosis

Venous thrombosis is the third most common vascular disease after acute myocardial infarction and stroke. There is a complex interplay of genetic and environmental risk factors which result in a VTE event (Souto et al. 2000). Its main clinical implication, DVT is a major preventable cause of morbidity and

mortality worldwide. During the past decades, extensive research has been carried out to identify and characterize the cellular and molecular mechanisms underlying VTE. It typically occurs in areas with decreased or mechanically reduced blood flow such as the pockets adjacent to valves in the deep veins of the leg, thus serving as a potential location for venous stasis. Rudolf Virchow's contribution in the form of Virchow's triad comprising of risk factors such as reduction in blood flow; changes in blood viscosity; and damage or abnormality in the vessel wall together determine the risk of venous thrombosis (Kumar et al. 2010). The occurrence of VTE is ~96% times in the lower extremities and ~4% in the upper extremities (Muñoz et al. 2008). Much as a result of the morbidity of DVT, the development of post-thrombotic syndrome occurs in up to 50% of patients within 2 years of DVT event, which includes a number of symptoms such as leg pain, swelling, and in severe cases, venous ulcers (Galanaud and Kahn 2014). Normal blood physiology maintains a delicate balance between pro- and anti-coagulant factors. Prevention, management, and treatment of VTE require understanding of its pathophysiology particularly for identification of high risk individuals, determining the dose and duration of anticoagulation treatment and warranting prophylactic measures.

22.3 VTE in Women

Ample number of studies has established that gender plays an important role in the incidence of venous thrombo-embolism. Both men and women are affected by DVT. But women's risk factors, such as being on birth control pills and being pregnant, can actually raise their chances of developing DVT during childbearing years. Thus women are at higher risk of VTE during fertile years, mainly due to the effects of pregnancy and oral contraceptive use (Bleker et al. 2014). From puberty to menopause, women bear the highest risk.

22.4 Important Observations

22.4.1 Alerts at Different Stages of Life!

1. *Estrogen-Containing Pills/oral contraceptives*
   Use of oral contraceptives is an established additional risk factor for venous thrombosis (Barsoum et al. 2010; Gomes and Deitcher 2004), and the effect is dependent both on the progestogen used and the dose of ethinylestradiol (Stegeman et al. 2013). Women who use oral contraceptives and have inherited clotting defects develop venous thrombosis not only more often but also sooner than do those without inherited clotting defects (Bloemenkamp et al. 2000). Estrogen-based medication, such as birth control pills and hormone therapy, increases risk of formation of blood clots by 2–5 times (Spitzer et al. 1996). All formulations of combined oral contraceptives (COCs) are associated with increased risk of thrombosis compared to non-users. Earlier till 1995, the risk

of VTE associated with contraception use was exclusively attributed to presence of ethinyl estradiol; therefore later, the dose of estrogen was lowered from 100 mcg to 35–50 mcg in the newer-generation pills. However, it was later observed that the type of progestin may also influence the risk of VTE. This was deduced because the use of third-generation COCs (containing desogestrel, drospirenone, or gestodene) was reported to result in a higher risk of VTE than the use of second-generation COCs (containing levonorgestrel) (Jick et al. 1995; Kemmeren et al. 2001). Gomes et al. also reported that first and third generation oral contraceptives possess a higher risk than second generation oral contraceptives (Gomes and Deitcher 2004). Contraception by injectable depot-medroxyprogesterone acetate is associated with threefold increase in VTE risk, whereas a levonorgestrel intrauterine device imparts no such risk (van Hylckama Vlieg et al. 2010). In retrospective cohort study of VTE recurrence, after incidence of first VTE occurring in women taking oral contraceptives, the incidence of recurrence in women who had stopped anticoagulation was 5.1% after 1 year and 14.2% after 5 years (Vaillant-Roussel et al. 2011). It is important for patients to know the risk of thrombosis before they decide to choose upon the oral contraceptives over unplanned pregnancy.

2. *Ovarian Stimulation*
   Ovarian hyperstimulation syndrome (OHSS) occurs in 1–10% of women undergoing ovarian hyperstimulation with exogenous gonadotrophin administration. It is a potentially life threatening complication (Brinsden et al. 1995). This syndrome has a wide clinical spectrum from minor abdominal discomfort, respiratory distress to thrombo-embolic event. In a case of 37 year old woman, it was suggested that a high index of suspicion for venous thrombosis is needed in women with ovarian hyper-stimulation syndrome (Chipwete et al. 2009). Ovarian stimulation cycles accompanying high serum estradiol levels, haemoconcentration, or OHSS are at potential risk of thrombo-embolism (Ou et al. 2003). This risk doubles after in vitro fertilization (IVF) and OHSS, in the first trimester. Sennström et al. (2017) suggested that IVF patients with OHSS can be prescribed with low-molecular-weight heparin during the first trimester, whereas other IVF patients should be given thromboprophylaxis based on the same risk factors as other pregnant women (Sennström et al. 2017).
3. *Pregnancy or Recent Birth*
   Pregnancy increases the risk of VTE four to fivefold over that in the non-pregnant state (Pomp et al. 2008a; Heit et al. 2005; Devis and Knuttinen 2017). During pregnancy, blood flow slows down, making it more likely to aggregate and form clots. Moreover, expanding uterus puts pressure on veins making it harder for blood to get through. Pulmonary embolism is a leading cause of maternal death during pregnancy and after childbirth. Heparin is an anticoagulant drug that's safe and often used during pregnancy. In women with past history of VTE, contrasting data is available on risk of recurrence during pregnancy (as reviewed by James et al. 2007). This information is of clinical importance, as it may decide upon whether or not women with previous thrombosis history should undergo VTE prophylaxis during pregnancy and after delivery (Palareti 2012)! A retrospective

study demonstrated that there is a temporary and a more than 3.5 fold increase in the relative risk of symptomatic recurrent thrombosis during pregnancy (Pabinger et al. 2002). However, another retrospective study concluded that significantly lower long-term risk of recurrent VTE exist in women with pregnancy-associated VTE, whereas women with unprovoked VTE have a higher risk of recurrent VTE during a subsequent pregnancy (White et al. 2008). Thus prophylactic administration of low molecular weight heparin (LMWH) during pregnancy and puerperium might reduce the risk of pregnancy-associated VTE.

4. *Hormone Replacement Therapy (HRT)*
   Exogenous hormone therapy (HT) is used by millions of women yearly as hormone contraception (HC) or postmenopausal hormone therapy (PHT). Antifibinolytic drugs, such as tranexamic acid are used for treatment of heavy menstrual bleeding (referred as menorrhagia). These drugs reduce bleeding by inhibition of endometrial clot-dissolving enzymes, however, they may increase the risk of developing blood clots in legs and lungs. Antifibinolytic drugs prevent the decomposition of fibrin in clotted blood, thus inhibiting dissolution of thrombi, making women more prone to thrombosis (Taparia et al. 2002). Oral HRT formulations can be estrogen only (unopposed) using conjugated equine estrogen or estradiol, or estrogen combined with a progestogen (opposed). Recent studies have confirmed that current users of HRT are at increased risk of venous thrombosis. Mahajan and coworkers presented a case of 43-year-old woman, who developed DVT after in vitro fertilization-oocyte donation (IVF-OD). They suggested that even a short-term use of HRT should be considered a risk factor for DVT, especially in the presence of additional risk factors such as obesity (Mahajan et al. 2015).

   Though it is established that COCs and HRT increases the risk of venous thrombosis, the data available on VTE recurrence in such subjects is less. A cumulative probability of VTE recurrence at 5 years after follow-up was reported to be 5.9% with a hormone-related first thrombosis compared to 4.3% in women of the same age with a first idiopathic event (Kyrle et al. 2004). In contrast to this, a posthoc analysis of the PREVENT study by Cushman et al. found that much of the lower rate of VTE recurrence in women versus men was explained by a lower recurrence risk among women with hormone-related thrombosis (46% lower risk than other women) (Cushman et al. 2006).

In contrast to the discussions so far, many research groups have independently reported that no sex specific differences occur in VTE occurrence or rather males are more prone to the disease. Roach and coworkers reviewed genetic and acquired risk factors for VTE in men and women and found no significant difference in prevalence or strength of factors like plaster cast immobilization, hospitalization, surgery, trauma, malignancy, hyperhomocyctenemia, factor V leiden, prothrombin G20210A, or blood group non O. They further summarized that the sex based differences in VTE occurrence can be attributed to X- or Y-linked mutations or mutation on a sex-specific gene (Roach et al. 2014a, b).

22.5 Medical Management of VTE

A cumulative effect of complex interaction between various acquired and inherited risk factors may trigger a VTE event. Treatment in early phases of DVT, help to curb fibrin clot formation and reduces the risk of progression of DVT to PE. The standard therapy for DVT is treatment with vitamin K-antagonists (VKAs) such as warfarin along with heparin or fractionated heparin bridging. However in past few years, large number of clinical trials has validated the use of direct oral anticoagulants (DOACs) in place of warfarin. DVT can be treated with oral anticoagulants alone, besides anticoagulation remains an important component of treatment. However, in cases of extensive thrombus burden, where rapid clot lysis is needed to reduce the risk of post-thrombotic syndrome, mechanical- and catheter-directed thrombolysis (CDT) may be used in the acute phase (Oklu and Wicky 2013). However, this thrombolytic therapy could be associated with an increased risk of major bleeding. During acute phase of thrombosis, which corresponds to the first 5–10 days of therapy, unfractioned heparin (UFH) or LMWH is utilized as a bridging agent when a VKA is planned. UFH has several advantages over LMWH as it has a shorter elimination half-life (~1 h) and its affects are fully reversible (Streiff et al. 2016), however substantial differences may occur in dosing requirement of different individuals depending upon their BMI, body weight, etc. Also, UFH has eight to tenfold increased risk for heparin-induced thrombocytopenia (HIT) when compared to LMWH (Linkins et al. 2012). Thus LMWH such as enoxaparin, Fondaparinux, a synthetic pentasaccharide is often the bridging therapy of choice. More recently, DOACs are considered as attractive alternative to VKAs as they have fewer drug-drug interactions and they can be taken orally. Some of these include dabigatran, rivaroxaban, apixaban, and edoxaban, however, each of them carries their own advantages and risks (As reviewed by Stone et al. 2017).

22.6 Lifestyle and Other Acquired Risk Factors Important for All Genders

Life style associated risk predictors of VTE include smoking and co-morbidities like hypertension, diabetes, abdominal obesity, and abnormal lipid profiles (Ageno et al. 2008; Pomp et al. 2008b; Deguchi et al. 2005). Smoking and obesity are very important risk factors for DVT, for both men and women. Thus, to minimize the risk of formation of sudden blood clots, leading a healthy lifestyle is strongly encouraged such as eating healthy diet, exercise, and maintaining a healthy body weight. Besides these, trauma, surgery or prolonged immobilization due to any reason is a risk factor for thrombotic complications. If a person has undergone major surgery or trauma, the risk of DVT is increased several times (Rogers 2001; White et al. 2003; Ilahi et al. 2005). Apart from these, advancing age is the key risk factor for DVT. The risk of developing DVT increases considerably after 50 years of age. In cases, advancing age is combined with inherited or genetic risk factors such as higher production of clotting factors or lower production of natural anticoagulants

in the body, the risk may increase several times. In such cases, prophylactic use of anticoagulants along with frequent monitoring of person is recommended.

Know the early signs of DVT/PE

- Blood clotting during DVT event usually occurs in the legs or arms. The limb gets swelled without any injury and pain and tenderness persists.
- The skin of the affected area might be warmer in touch and may appear red or discolored. The condition should not be ignored for longer duration as the clot might break off and travel to the lungs. This results in PE and can be life threatening.
- Signs and symptoms of PE include difficulty breathing, chest pain that worsens with a deep breath or cough, coughing up blood, and faster than normal or irregular heartbeat.
- One should immediately seek medical attention when these signs are experienced.

Preventing DVT

- Avoid long immobilization period: One should try to move around as soon as possible after surgery, illness, or injury.
- Avoid long hours of sitting and still travelling: A few minutes of limbs movement after long constant sitting is recommended to avoid DVT. Also, one should try to exercise legs, such as moving heels and toes and wear loose-fitting clothes during long flights.
- Be aware for familial risk or previous history of clots: A person with familial or past history of blood clots/thrombotic events is more likely to develop VTE or its recurrence. One should be more cautious in such cases.
- Maintain healthy lifestyle: DVT risk can be minimized by maintaining a healthy weight, avoiding a sedentary lifestyle, balancing lipid profile, and no smoking.

22.7 Summary

A thorough understanding of underlying epidemiology and associated risk factors of VTE is extremely important for its appropriate management, diagnosis, and treatment modalities. DVT is a serious condition which may lead to PE and eventually death. It is often underdiagnosed; however it is completely preventable and treatable if discovered early. The detailed mechanism of gender-specific manifestations of venous thrombosis is still unknown. Though several studies have shown that women with gender-specific risk factors such as oral contraceptives, pregnancy, puerperium, and hormonal replacement therapy pose higher risk of venous thrombosis/DVT, the results are contradictory. This medical condition needs more attention and awareness in the general population to minimize the causalities related to it. Women,

particularly, need to understand risks and benefits associated with various treatments undertaken from the age of puberty till menopause.

References

Ageno W, Becattini C, Brighton T, Selby R, Kamphuisen PW (2008) Cardiovascular risk factors and venous thromboembolism: a meta-analysis. Circulation 117:93–102

Alotaibi GS, Wu C, Senthilselvan A, McMurtry MS (2016) Secular trends in incidence and mortality of acute venous thromboembolism: the AB-VTE population-based study. Am J Med 129(8):879.e19–879.e25

Anderson FA Jr, Wheeler HB, Goldberg RJ, Hosmer DW, Patwardhan NA, Jovanovic B, Forcier A, Dalen JE (1991) A population-based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism: the Worcester DVT Study. Arch Intern Med 151:933–938

Barnes PM, Adams PF, Powell-Griner E (2008) Health characteristics of the Asian adult population: United States, 2004–2006. Adv Data 1–22

Barsoum MK, Heit JA, Ashrani AA, Leibson CL, Petterson TM, Bailey KR (2010) Is progestin an independent risk factor for incident venous thromboembolism? A population-based case-control study. Thromb Res 126:373–378

Blann AD, Lip GY (2001) Virchow's triad revisited: the importance of soluble coagulation factors, the endothelium, and platelets. Thromb Res 101(4):321–327

Bleker SM, Coppens M, Middeldorp S (2014) Sex, thrombosis and inherited thrombophilia. Blood Rev 28(3):123–133

Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Vandenbroucke JP (2000) Higher risk of venous thrombosis during early use of oral contraceptives in women with inherited clotting defects. Arch Intern Med 160(1):49–52

Brinsden PR, Wada I, Tan SL, Balen A, Jacobs HS (1995) Diagnosis, prevention and management of ovarian hyperstimulation syndrome. Br J Obstet Gynaecol 102(10):767–772

Chipwete SE, Bugren S, Rafla N (2009) Thrombosis post ovarian hyperstimulation. Fertil Steril 91(5):1956.e13–1956.e14

Christiansen SC, Lijfering WM, Helmerhorst FM, Rosendaal FR, Cannegieter SC (2010) Sex difference in risk of recurrent venous thrombosis and the risk profile for a second event. J Thromb Haemost 8(10):2159–2168

Coutinho JM, Ferro JM, Canhão P, Barinagarrementeria F, Cantú C, Bousser MG, Stam J (2009) Cerebral venous and sinus thrombosis in women. Stroke 40(7):2356–2361

Cushman M, Glynn RJ, Goldhaber SZ, Moll S, Bauer KA, Dietcher S, Shrivastava S, Ridker PM (2006) Hormonal factors and risk of recurrent venous thrombosis: the Prevention of Recurrent Venous Thromboembolism trial. J Thromb Haemost 4(10):2199–2203

De Stefano V, Finazzi G, Mannucci PM (1996) Inherited thrombophilia: pathogenesis, clinical syndromes, and management. Blood 87(9):3531–3544

Deguchi H, Pecheniuk NM, Elias DJ, Averell PM, Griffin JH (2005) High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. Circulation 112: 893–899

Delluc A, Tromeur C, Le Ven F, Gouillou M, Paleiron N, Bressollette L, Nonent M, Salaun PY, Lacut K, Leroyer C, Le Gal G, Couturaud F, Mottier D, EPIGETBO Study Group (2016) Current incidence of venous thromboembolism and comparison with 1998: a community-based study in Western France. Thromb Haemost 116(5):967–974

Devis P, Knuttinen MG (2017) Deep venous thrombosis in pregnancy: incidence, pathogenesis and endovascular management. Cardiovasc Diagn Ther 7(Suppl 3):S309–S319

Douketis J, Tosetto A, Marcucci M, Baglin T, Cosmi B, Cushman M, Kyrle P, Poli D, Tait RC, Iorio A (2011) Risk of recurrence after venous thromboembolism in men and women: patient level meta-analysis. Br Med J 342:d813

Galanaud JP, Kahn SR (2014) Postthrombotic syndrome: a 2014 update. Curr Opin Cardiol 29(6): 514–519

Gomes MP, Deitcher SR (2004) Risk of venous thromboembolic disease associated with hormonal contraceptives and hormone replacement therapy: a clinical review. Arch Intern Med 164:1965–1976

Heit JA, Kobbervig CE, James AH, Petterson TM, Bailey KR, Melton LJ 3rd (2005) Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. Ann Intern Med 143(10):697–706

Heit JA, Spencer FA, White RH (2016) The epidemiology of venous thromboembolism. J Thromb Thrombolysis 41(1):3–14

Ilahi OA, Reddy J, Ahmad I (2005) Deep venous thrombosis after knee arthroscopy: a meta-analysis. Arthroscopy 21:727–730

James AH, Grotegut CA, Brancazio LR, Brown H (2007) Thromboembolism in pregnancy: recurrence and its prevention. Semin Perinatol 31(3):167–175

Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C (1995) Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet 346(8990):1589–1593

Johansson M, Johansson L, Lind M (2014) Incidence of venous thromboembolism in northern Sweden (VEINS): a population-based study. Thromb J 12(1):6

Jun ZJ, Ping T, Lei Y, Li L, Ming SY, Jing W (2006) Prevalence of factor V Leiden and prothrombin G20210A mutations in Chinese patients with deep venous thrombosis and pulmonary embolism. Clin Lab Haematol 28:111–116

Kemmeren JM, Algra A, Grobbee DE (2001) Third generation oral contraceptives andrisk of venous thrombosis: meta-analysis. BMJ 323(7305):131–134

Koster T, Blann AD, Briet E, Vandenbroucke JP, Rosendaal FR (1995) Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet 345(8943): 152–155

Kumar DR, Hanlin E, Glurich I, Mazza JJ, Yale SH (2010) Virchow's contribution to the understanding of thrombosis and cellular biology. Clin Med Res 8(3–4):168–172

Kyrle PA, Minar E, Bialonczyk C, Hirschl M, Weltermann A, Eichinger S (2004) The risk of recurrent venous thromboembolism in men and women. NEJM 350(25):2558–2563

Laczkovics C, Grafenhofer H, Kaider A, Quehenberger P, Simanek R, Mannhalter C, Lechner K, Pabinger I (2007) Risk of recurrence after a first venous thromboembolic event in young women. Haematologica 92(9):1201–1207

Linkins LA, Dans AL, Moores LK, Bona R, Davidson BL, Schulman S, Crowther M (2012) Treatment and prevention of heparin-induced thrombocytopenia: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 141(2 Suppl):e495S–e530S

Linnemann B, Zgouras D, Schindewolf M, Schwonberg J, Jarosch-Preusche M, Lindhoff-Last E (2008) Impact of sex and traditional cardiovascular risk factors on the risk of recurrent venous thromboembolism: results from the German MAISTHRO Registry. Blood Coagul Fibrinolysis 19(2):159–165

Mahajan N, Naidu P, Gupta S, Rani K (2015) Deep venous thrombosis in a patient undergoing In-vitro fertilization with oocyte donation. J Hum Reprod Sci 8(3):182–185

Molina JA, Jiang ZG, Heng BH, Ong BK (2009) Venous thromboembolism at the National Healthcare Group, Singapore. Ann Acad Med Singap 38(6):470–478

Muñoz FJ, Mismetti P, Poggio R, Valle R, Barrón M, Guil M, Monreal M (2008) RIETE Investigators. Clinical outcome of patients with upper-extremity deep vein thrombosis: results from the RIETE Registry. Chest 133(1):143–148

Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrøm J (2007) Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 5: 692–699

Nordström M, Lindblad B, Bergqvist D, Kjellström T (1992) A prospective study of the incidence of deep-vein thrombosis within a defined urban population. J Intern Med 232(2):155–160

Oger E (2000) Incidence of venous thromboembolism: a community-based study in Western France: EPI-GETBP Study Group: Groupe d'Etude de la Thrombose de Bretagne Occidentale. Thromb Haemost 83:657–660

Oklu R, Wicky S (2013) Catheter-directed thrombolysis of deep venous thrombosis. Semin Thromb Hemost 39(4):446–451

Ota S, Yamada N, Ogihara Y, Tsuji A, Ishikura K, Nakamura M, Wada H, Ito M (2011) High plasma level of factor VIII: an important risk factor for venous thromboembolism. Circ J 75(6): 1472–1475

Ou YC, Kao YL, Lai SL, Kung FT, Huang FJ, Chang SY, ChangChien CC (2003) Thromboembolism after ovarian stimulation: successful management of a woman with superior sagittal sinus thrombosis after IVF and embryo transfer: case report. Hum Reprod 18(11):2375–2381

Pabinger I, Grafenhofer H, Kyrle PA, Quehenberger P, Mannhalter C, Lechner K, Kaider A (2002) Temporary increase in the risk for recurrence during pregnancy in women with a history of venous thromboembolism. Blood 100(3):1060–1062

Palareti G (2012) Recurrent venous thromboembolism: what is the risk and how to prevent it. Scientifica 2012:391734

Pomp ER, Lenselink AM, Rosendaal FR, Doggen CJ (2008a) Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study. J Thromb Haemost 6(4):632–637

Pomp ER, Rosendaal FR, Doggen CJ (2008b) Smoking increases the risk of venous thrombosis and acts synergistically with oral contraceptive use. Am J Hematol 83:97–102

Roach REJ, Cannegieter SC, Lijfering WM (2014a) Differential risks in men and women for first and recurrent venous thrombosis: the role of genes and environment. J Thromb Haemost 12(10): 1593–1600

Roach REJ, Lijfering WM, Rosendaal FR, Cannegieter SC, le Cessie S (2014b) Sex difference in risk of second but not of first venous thrombosis paradox explained. Circulation 129:51–56

Rogers FB (2001) Venous thromboembolism in trauma patients: a review. Surgery 130:1–12

Rosendaal FR (2005) Venous thrombosis: the role of genes, environment, and behavior. Hematology Am Soc Hematol Educ Program 1–12

Rosendaal FR, Reitsma PH (2009) Genetics of venous thrombosis. J Thromb Haemost 7(Suppl 1): 301–304

Sennström M, Rova K, Hellgren M, Hjertberg R, Nord E, Thurn L, Lindqvist PG (2017) Thromboembolism and in vitro fertilization—a systematic review. Acta Obstet Gynecol Scand 96(9): 1045–1052

Silverstein MD, Heit JA, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ 3rd (1998) Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. Arch Intern Med 158:585–593

Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, Coll I, Felices R, Stone W, Fontcuberta J, Blangero J (2000) Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic analysis of idiopathic thrombophilia. Am J Hum Genet 67(6):1452–1459

Spencer FA, Emery C, Joffe SW, Pacifico L, Lessard D, Reed G, Gore JM, Goldberg RJ (2009) Incidence rates, clinical profile, and outcomes of patients with venous thromboembolism: the Worcester VTE study. J Thromb Thrombolysis 28:401–409

Spitzer WO, Lewis MA, Heinemann LA, Thorogood M, MacRae KD (1996) Third generation oral contraceptives and risk of venous thromboembolic disorders: an international case-control study. Transnational Research Group on Oral Contraceptives and the Health of Young Women. BMJ 312:83–88. Level II-2

Stegeman BH, de Bastos M, Rosendaal FR, van HylckamaVlieg A, Helmerhorst FM, Stijnen T, Dekkers OM (2013) Different combined oral contraceptives and the risk of venous thrombosis: systematic review and network meta-analysis. BMJ 347:f5298

Stone J, Hangge P, Albadawi H, Wallace A, Shamoun F, Knuttien MG, Naidu S, Oklu R (2017) Deep vein thrombosis: pathogenesis, diagnosis, and medical management. Cardiovasc Diagn Ther 7(Suppl 3):S276–S284

Streiff MB, Agnelli G, Connors JM, Crowther M, Eichinger S, Lopes R, McBane RD, Moll S, Ansell J (2016) Guidance for the treatment of deep vein thrombosis and pulmonary embolism. J Thromb Thrombolysis 41(1):32–67

Taparia M, Cordingley FT, Leahy MF (2002) Pulmonary embolism associated with tranexamic acid in severe acquired haemophilia. Eur J Haematol 68(5):307–309

Trinchero A, Scheres LJJ, Prochaska JH, Ambaglio C, Wild PS, Middeldorp S, Konstantinides SV, Barco S (2018) Sex-specific differences in the distal versus proximal presenting location of acute deep vein thrombosis. Thromb Res 172:74–79

Vaillant-Roussel H, Ouchchane L, Dauphin C, Philippe P, Ruivard M (2011) Risk factors for recurrence of venous thromboembolism associated with the use of oral contraceptives. Contraception 84:e23–e30

van Hylckama Vlieg A, Helmerhorst FM, Rosendaal FR (2010) The risk of deep venous thrombosis associated with injectable depot-medroxyprogesterone acetate contraceptives or a levonorgestrel intrauterine device. Arterioscler Thromb Vasc Biol 30:2297–2300

White RH, Zhou H, Romano PS (2003) Incidence of symptomatic venous thromboembolism after different elective or urgent surgical procedures. Thromb Haemost 90:446–455

White RH, Zhou H, Murin S, Harvey D (2005) Effect of ethnicity and gender on the incidence of venous thromboembolism in a diverse population in California in 1996. Thromb Haemost 93: 98–305

White RH, Chan WS, Zhou H, Ginsberg JS (2008) Recurrent venous thromboembolism after pregnancy-associated versus unprovoked thromboembolism. Thromb Haemost 100(2):246–252

Zakai NA, McClure LA (2011) Racial differences in venous thromboembolism. J Thromb Haemost 9:1877–1882

Biomedical Science and Women's Health

23

Vanita Suri and Ritu Aggarwal

Abstract

Health is a universal right. It is the duty of every nation to take care of its women and girls as their health is the health of population. There are gender-related differences when we discuss women's health issues. Therefore, it is important to discuss the role of biomedical sciences in women's health. Whether it is related to diagnostics or therapeutics, scientific advances have created wonders in this area. Pregnancy testing, prenatal diagnostics, and prediction of preeclampsia occurrence and prognosis are dependent upon biomedical sciences. Endometriosis and gynecological malignancies are other broad and incompletely understood areas where there is a lot of scope for exploration with the help of molecular technologies. In this chapter, we have tried to review the relationship between women's health issues and biomedical sciences.

Keywords

Women's health · Biomedical science · Pregnancy · Prenatal diagnosis · Cell-free DNA · Aneuploidy · Fetal medicine · Karyotyping · Microarray · Preeclampsia · Endometriosis · Human papillomavirus · HPV vaccine · Toll-like receptors · Carcinoma · PCOS

V. Suri (✉)
Department of Obstetrics and Gynecology, PGIMER, Chandigarh, India, Chandigarh

R. Aggarwal
Department of Immunopathology, PGIMER, Chandigarh, Chandigarh, India

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_23

23.1 Introduction

Science and health are inseparable. Biomedical science has totally transformed the experience of health issues arising in a woman's life, whether they are gender related or age related. In this chapter, we will try to review the impact of biomedical innovations on specific areas related to women's health.

23.2 Diagnosis of Pregnancy

Let us start with the beginning. The twentieth century has witnessed the medicalization of pregnancy. Biotechnology has transformed the experience of pregnancy from home delivery to hospital delivery, from clinical examination to ultrasound, and from neonatal surprises to prenatal diagnosis and therapy. The new technologies consisting of obstetrical ultrasound, study of chromosomes in the amniotic fluid by amniocentesis and by chorionic villus biopsy have revolutionized the obstetric practice (Salim Al-Gailani and Angela Davis 2014; Cariati et al. 2019).

Testing for pregnancy dates back to 1350 BCE, according to an ancient Egyptian papyrus. The physicians believed that the urine of pregnant women will lead to germination of wheat and barley grains. On testing its authenticity, it was found that the test was positive in 70% of women.

In the 1920s, biomedical science finally came in picture with the identification of a specific hormone of pregnancy which was named as human chorionic gonadotropin (hCG). Selmar Aschheim and Bernhard Zondek introduced a pregnancy test based on the presence of this hormone in the urine of pregnant females in 1927. In the beginning, the urine of pregnant women used to be injected in immature mice, and the oestrous effect on their ovaries would detect the presence of pregnancy hormone, i.e., hCG. These tests were cumbersome and led to animal slaughter, which was not ethical (Gnoth and Johnson 2014).

In 1960, Leif Wide and Carl Gemzell developed a haem agglutination inhibition test. In this immunoassay purified hCG was mixed with a urine sample and antibodies directed against hCG. The test though cheaper and rapid had very less sensitivity.

Further research led to the commercially available 2 min pregnancy test which anybody can do at home and has 99% accuracy. Thus molecular techniques made the pregnancy diagnosis a home-based simple technique.

23.3 Fetal Medicine

Karyotyping and microarray-based chromosomal analysis are the recent prenatal diagnosis (PND) strategies to detect chromosomal alterations during pregnancy (Wellesley et al. 2012).

Microarray-based chromosomal evaluation has helped in increasing the diagnostic yield of prenatal tests; thus, it is recommended as a first-level test when

ultrasound detects some markers for aneuploidy. This technique helps in diagnosis of chromosomal aneuploidy, microdeletions, and microduplications within a chromosome not detectable by a standard karyotype *(Practice bulletin no. 163).*

The traditional prenatal diagnostic techniques will be soon replaced by more sensitive techniques like NGS and targeted sequencing of single disease causing genes or group of genes, prenatal exome sequencing and RNA-sequencing (Hu et al. 2018; Rasmussen et al. 2018).

The feasibility of whole genome sequencing (WGS) in prenatal settings is lower than ES due to variants interpretation and higher cost. RNA-Seq studies on fetal tissues have the potential to help in understanding the fetal development and mechanisms of specific diseases of interest; however, currently they may be useful just for research purposes only (Vora and Hui 2018; Mao et al. 2018).

The free fetal DNA (cffDNA) is present in the maternal blood. It was analyzed in 1997 for the first time when Y chromosome was seen in the plasma of some women with male fetus. The analysis of cffDNA is the most recent and innovative prenatal screening test for chromosomal abnormalities, CNVs, and microdeletion. Non-invasive prenatal testing (NIPT) is used for screening, testing, or diagnosis of fetal chromosomal or genetic anomalies by analyzing directly cffDNA in maternal plasma or serum (Wong and Lo 2016; Lo 2013). The NGS-based analysis of the whole cffDNA present in the maternal plasma is carried out (Ong et al. 2013). Several algorithms and different techniques are used for diagnosing aneuploidy in the fetal cells (Hudecova et al. 2014; Tiller et al. 2015). Other genetic disorders for which circulating single molecule amplification and resequencing technology (cSMART) technique have been used are Phenylketonuria and Wilson disease (Duan et al. 2019; Lv et al. 2015). Recently, high sensitivity and specificity have been shown by combining NGS with the quantitative counting of the template (input DNA) for the NIPT of the most common genetic disorders (sickle cell disease, spinal muscular atrophy, cystic fibrosis, and thalassemias) (Tsao et al. 2019). The future sees the possibility of studying cell-free RNA in amniotic fluid as well as in maternal serum for prenatal diagnosis (Vora and Hui 2018).

23.4 Pregnancy Complications

23.4.1 Preeclampsia

Preeclampsia is a devastating pregnancy-associated disorder characterized by the onset of hypertension, proteinuria, and edema. Preeclampsia affects about 5–8% of all pregnant women. Despite intensive investigation, our current understanding of the pathophysiology is limited. Certain biomarkers have been identified which are secreted by placenta in response to ischemia and hypoxia. One of these is sFlt1. Vuorela and colleagues from Finland, in 2000 reported that sFlt1 is significantly elevated in the amniotic fluid of preeclamptic women. It is thought that excess sFlt1 neutralizes both free-VEGF and free–placental growth factor (PlGF) in maternal circulation, leading to endothelial damage and the onset of this multisystem disorder

(Sugimoto et al. 2003; Maynard et al. 2003). Based on this information, lot of research is being carried out on the role of sFlt1 and PIGF in the diagnosis, as well as prognosis of preeclampsia.

Maternal immune response against fetus and placenta may be responsible for development of preeclampsia. Normally Th2 response is seen during pregnancy, but in preeclampsia Th1 response dominates which may be due to activation of natural killer cells (NK cells). Thus NK cells derived Th1 cytokines may cause all the inflammatory changes of preeclampsia and endothelial damage leading to multisystem organ involvement.

Catechol-O-methyltransferase (COMT) converts 17-hydroxyestradiol into 2-methoxyestradiol (2-ME), as a rate-limiting step in estrogen breakdown (Kanasaki et al. 2008). During pregnancy the levels of 2-ME increase but on the other hand the plasma levels of this factor in preeclamptic women are suppressed. COMT suppression was first described in 1988 by Barnea et al. The activity of the COMT enzyme displays a tri-modal frequency distribution in human populations because of the presence of a functional polymorphism in the coding sequence. This functional *COMT* polymorphism is associated with fetal growth restriction and abnormalities (Tunbridge et al. 2006; Sata et al. 2006). Many drugs can also lead to COMT suppression thus raising concern regarding their use in preeclampsia. Hydralazine and alpha methyldopa have been implicated in causing COMT suppression and may lead to drug exacerbated preeclampsia (Barnea et al. 1986). More work is required in this field before we start using these biomarkers for diagnosing and managing women with preeclampsia as a standard of care.

23.4.2 Acute Fatty Liver of Pregnancy (AFLP)

Acute fatty liver of pregnancy is an acute emergency. Though rare, but it is a fatal disease for the mother. The pathogenesis of AFLP is not properly understood but defects in the fatty acid metabolism appear to play some role. Due to some enzyme deficiency the defective metabolism of fatty acids takes place and the intermediate products cause damage to the maternal hepatocytes causing havoc to maternal and fetal life (Ibdah et al. 1999). Around 20% of AFLP is caused by deficiency of fetal long-chain 3-hydroxyacyl CoA dehydrogenase (LCHAD) which catalyzes a step in beta-oxidation of mitochondrial fatty acids in which 3-ketoacyl-CoA is formed from 3-hydroxyacyl-CoA (Tran et al. 2016). In fetuses homozygous for LCHAD deficiency, the fetoplacental unit cannot perform this step, so levels of intermediate products of fatty acid metabolism increase and enter the maternal circulation (Yang et al. 2002; Natarajan and Ibdah 2018) and this contributes to long-chain metabolites accumulating in maternal blood and hepatocytes, resulting in toxic effects. The homozygous G1528C mutation, which alters amino acid 474 from glutamic acid to glutamine on the protein (E474Q), seems to be the most common genotype associated with development of AFLP (Ibdah et al. 1999). Sometimes, it is very difficult to differentiate HELLP syndrome from AFLP. This understanding of

enzyme deficiency and fatty acid metabolism may contribute further to our understanding of etiology of preeclampsia and its complications.

23.4.3 Gynecological Oncology

Biomedical research has brought about astonishing achievements in the field of gynecological oncology. One, which is of paramount importance, is the unravelling of the high-risk human papillomavirus as the etiologic agent for cervical cancer. It was towards the end of the twentieth century that studies on the possible role of human papillomavirus were initiated. Zur Hausen made a significant research contribution in the field of oncoviruses. In the year 1976, it was for the first time that he published his observations that human papillomavirus plays a significant role in the causation of cervical cancer (zur Hausen 1976). Subsequently, along with other co-workers, he recognized HPV16 and HPV18 in cervical carcinoma in the year 1983–1984. Zur Hausen used nucleic acid hybridization in the tissue samples to identify the Human Papillomavirus (Durst et al. 1983; Boshart et al. 1984). The research outcome made an immense contribution to the vaccine development, which enabled the vaccine being available in 2006. He was awarded the Nobel Prize for medicine for his contribution in the field of cervical carcinoma.

The development of the HPV prophylactic vaccine was a major innovation for the prevention of carcinoma of the cervix. HPV prophylactic vaccine is the first of its kind, which will prove a game-changer for cervical cancer in the years to come. Cervical cancer which is responsible for a significant number of deaths among gynecological cancers, is now preventable. The first prophylactic cancer vaccine, Gardasil, was a quadrivalent vaccine that received approval by the U.S. Food and Drug Administration in 2006. The scope of the vaccine included the prevention of cervical cancer, precancerous genital lesions, and genital warts, which occurred due to infection with HPV6, HPV11, HPV16, and HPV18 (Yugawa et al. 2007). Subsequently, Gardasil®9, which prevents infection against the nine HPV subtypes 6/11/16/18/31/33/45/52/58, was approved for coverage with additional subtypes. In 2009, another vaccine, Cervarix, which is a prophylactic vaccine that prevented infection from HPV 16 and 18 subtypes, received a nod by the FDA to prevent cervical carcinoma and other precancerous lesions, which occurred due to the human papillomavirus (HPV) types 16 and 18. All the vaccines mentioned above were useful in the prevention of HPV infections caused by the targeted HPV subtypes by inciting the formation of neutralizing antibodies, which prevents the entry of the viral particles into the host tissue (Joura et al. 2015).

23.4.4 Novel Vaccine Adjuvants

At present, research and development are centered around the TLR ligands for their potential role as novel vaccine adjuvants. Strategies which target TLR signalling pathways have been targeted in clinical practice to augment the generation of the

immunogenic response of the DNA vaccines and at the same time augment the efficiency of the T lymphocyte in combating the viral infection as well as to halt the inflammatory response, which results from bacterial infections (Hedayat et al. 2011). Experiments have demonstrated that simultaneous triggering of multiple pathways of TLRs by vaccines induced an immunogenic response, which was perceived as better. At present, the three TLR agonists, which have the approval of the international regulatory agencies for use in cancer patients, are monophosphoryl lipid A (MPL), bacillus Calmette-Guérin (BCG), and imiquimod (Mata-Haro et al. 2007; Hemmi et al. 2002). Akin to the LPS, MPL is known to activate the TRAM and TRIF signaling pathways, reducing the MYD-88 dependent signal pathway, which promotes inflammatory changes (Mata-Haro et al. 2007). The clinical trials which employed the CpG ODNs as the constituent of immunotherapy in patients with cancer suggested that CpG ODN alone or in combination with chemotherapy, can lead to a potent anti-tumor immune response that translates to clinical benefit (Hemmi et al. 2002). Adjuvant systems that use TLR adjuvants in different combinations, including alum, MPL, and CpG ODN, have exhibited superior efficacy compared to a mono TLR adjuvant. TLRs, when employed as molecular adjuvants, provide a novel target for HPV infection prevention and promote the concerted efforts for efficient vaccine development (Manegold et al. 2008; Garcon et al. 2007).

Here we would like to present our seminal work on the Toll-like receptors expression and the downstream signaling pathways in the cancer of the cervix.

Toll-like receptors are a vital element of the innate immune mechanisms. HPV is well known as an etiological factor of cervical cancer and is known to impact the gene expression of the TLRs and the subsequent downstream signalling pathway. It, however, remains ill-understood whether the HPV can cause the modulation of the expression of TLRs. We studied the relative gene expression of TLRs and the signalling pathway, which were downstream in tissues of women with carcinoma of the cervix, which tested positive for HPV. The experiments were performed on fresh tissues of the squamous cell carcinoma of the cervix (CSCC) and tissue from the normal cervix. The experiments involved analyzing the mRNA gene expression profile with the PCR Array. The expression of the translated proteins of some of the relevant genes was studied with the western blot technique. In addition, the cervicovaginal washes were analyzed for the cytokine levels using a Luminex multiplex platform. We recorded a significantly upregulated expression of TLR4 and downregulation of TLR2,7 in the cervical tissue with HR-HPV infection.

On the other hand, we observed the low expression of TLR4,7 in CSCC. The genes of cancer allied pathways, RELA, AKT, CDKN2A, and MDM2, exhibited an upregulated expression in cancer cervix. The data on protein expression corroborated with the observation of the gene expression profile. Dwindling levels of the Th1 cytokines TNF-α, IFN-ɣ, IL-17, and IL-12 in the Carcinoma cervix was noted. The levels of IL-1β, IL-6, and IL-2 were significantly increased in HR-HPV infected cervix. When we studied the disease-free survival in our cohort, the high expression of TLR4 and low TLR7 expression were associated with poor prognosis, as depicted by the Kaplan Meier curve (Guleria et al. 2019). In a previous study, we

reported that a significant downregulation in the relative gene expression of TLR3 ($p < 0.0001$), TLR4 ($p < 0.0005$), and TLR5 ($p < 0.0001$) was observed in cancer cervix (Aggarwal et al. 2015). The findings prompt us to think that HPV downregulated the innate immune mechanisms of the host to persist.

There has been an astronomical rise in the availability of cancer immunotherapeutic agents in the last decade. The advent of the new class of cancer therapeutics, the immune checkpoint inhibitor, likely provides a promising outcome for gynecological cancer. In contrast to cytotoxic chemotherapy, which primarily targets mechanisms for cellular growth and division, the checkpoint inhibitors do not target tumor cells directly. Instead, the checkpoint inhibitors block ligand/receptor interactions, responsible for dampening the immune response to the tumor (Pardoll 2012). Several immune checkpoints regulate the immune system. These pathways are vital for self-tolerance, such that the immune system refrains from attacking its cells. However, some cancers stimulating immune checkpoint targets and evade an attack on themselves (Pardoll 2012).

Inhibitory checkpoint molecules are components of cancer immunotherapy which are potential therapeutic targets in several cancers. Currently, the checkpoint inhibitors which are approved are the blockers of CTLA-4 and PD-1 and PD-L1 (Butte et al. 2007). For these remarkable fundamental scientific discoveries, James P. Allison and TasukuHonjo won the Nobel Prize in Physiology or Medicine in 2018. Several immune check points are currently being targeted in oncology, but the most notable among these are PDL1/PD-1 (durvaluemab, avelumab, atezolizumab/pembrolizumab, nivolumab, cemiplimab) and CTLA-4 (ipilimumab, tremelimumab). Targeting of many other novel checkpoints is currently in development. Many of these pathways are active in parallel, occurring at both the level of the tumor as well as more centrally within lymph nodes (Kurnit et al. 2020).

As the role of immuno-oncology continues to grow, the role of these agents in the therapy for women with gynecologic malignancies will broaden in the coming days. Not only are novel immune checkpoint inhibitors emerging, but other novel agents targeting the immune system are in the pipeline and being developed at a rapid pace. Novel immuno-oncology agents are being analyzed in the initial phase of several clinical trials. Drugs targeting the stimulator of interferon genes (STING) pathway, oncologic vaccines, and adoptive cell therapy (e.g., chimeric antigen receptor [CAR] T cells, tumor-infiltrating lymphocytes [TILs]) are currently under investigation in clinical trials (Kurnit et al. 2020).

Given the therapeutic efficacy seen in several gynecologictumors with checkpoint inhibitors, it can be hypothesized that these novel agents may come to the rescue of several women with gynecological malignancy. As these agents become more widely used, it will generate demand for understanding and critically analyzing the side effect profiles associated with each of these newer classes before incorporating these are inducted into regular practice (Kurnit et al. 2020).

Currently, many exciting cooperative group trials are underway in the United States: GY-018 is evaluating pembrolizumab in the frontline setting with maintenance pembrolizumab for endometrial cancer patients, GY-021 is looking at the combination of tremelimumab with olaparib for patients with platinum-sensitive

ovarian cancer upfront and GY-020, and GOG-3047 are assessing the combination of chemoradiation with pembrolizumab followed by maintenance as the frontline treatment of endometrial and cervical cancer patients, respectively (Kurnit et al. 2020).

23.5 Other Conditions

23.5.1 PCOS

PCOS affects majority of women during puberty and has lifelong consequences. Exact etiopathogenesis of this syndrome is not clear but few genes have been identified which are responsible for this condition. A wide variety of genetic variants with linkage to PCOS have been identified by candidate gene and molecular genetic studies (Rosenfield and Ehrmann 2016).The candidate gene approach led to the discovery of coding and regulatory variants in the AMHR (AMH receptor) genes and AMH (anti-müllerian hormone) in 6.7% of PCOS patients (Gorsic et al. 2019). Genome-wide association studies (GWAS) have been carried out to identify genes linked to PCOS. A meta-analysis of GWAS studies identified 13 loci common to all PCOS diagnostic criteria that were also associated with hyperandrogenism, gonadotropin levels, and testosterone levels, as well as the metabolic traits associated with PCOS (Day et al. 2018).

23.5.2 Endometriosis

Endometriosis is commonly seen in women of reproductive age group and is prevalent in 10–15% of menstruating women and 35% of infertile women (Olive and Schwartz 1993; Klemmt and Starzinski-Powitz 2018).

The etiology and pathogenesis of endometriosis are still not clear and various theories have been postulated. This area is another fertile ground for molecular research. Various cytokines and other inflammatory markers have been linked to its pathogenesis. Tumor necrosis factor alpha has been associated with increased proliferative potential of endometrial cells in places other than uterus (Iwabe et al. 2000). There is evidence of germline predisposition as well as role of epigenetics (Kennedy et al. 1995; Yano et al. 1999).

23.6 Summary

Biomedical sciences have revolutionized the approach to women's health problems, ranging from diagnostics to therapeutics. Knowledge about different complications of pregnancy which are major contributors to maternal mortality is still incomplete. There is a lot of scope for research in areas of preeclampsia and eclampsia, AFLP, recurrent pregnancy losses, endometriosis, PCOS, fibroids, and gynecological

malignancy. We desperately need answers to the differential response of patients to the similar events of pregnancy or some disease and biomedical sciences have the potential to answer these questions.

References

Aggarwal R, Misra S, Guleria C et al (2015) Characterization of Toll-like receptor transcriptome in squamous cell carcinoma of cervix: a case-control study. Gynecol Oncol 138:358–362

Barnea ER, Fakih H, Oelsner G et al (1986) Effect of antihypertensive drugs on catechol-O-methyltransferase and monoamine oxidase activity in human term placental explants. Gynecol Obstet Invest 21:124–130

Barnea ER, MacLusky NJ, DeCherney AH et al (1988) Catechol-o-methyl transferase activity in the human term placenta. Am J Perinatol 5:121–127

Boshart M, Gissman L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H (1984) A new type of papillomavirus DNA, its presence in genital cancer and in cell lines derived from genital cancer. EMBO J 3:1151–1157

Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ (2007) Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 27:111–122

Cariati F, D'Argenio V, Tomaiuolo R (2019) Innovative technologies for diagnosis and screening of genetic diseases in antenatal age. https://doi.org/10.21037/jlpm.2019.11.02

Day F, Karaderi T, Jones MR et al (2018) Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria. PLoS Genet 14:e1007813

Duan H, Liu N, Zhao Z et al (2019) Non-invasive prenatal testing of pregnancies at risk for phenylketonuria. Arch Dis Child Fetal Neonatal Ed 104:F24–F29

Durst M, Gissmann L, Ikenberg H, zur Hausen H (1983) A papillomavirus DNA from a cervical carcinoma and its prevalence in cervical biopsy samples from different geographic regions. Proc Natl Acad Sci U S A 80:3812–3815

Garcon N, Chomez P, Van Mechelen M (2007) GlaxoSmithKline adjuvant systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 6:723–739

Gnoth C, Johnson S (2014) Strips of hope: accuracy of home pregnancy tests and new developments. Geburtshilfe Frauenheilkd 74(7):661–669

Gorsic LK, Dapas M, Legro RS et al (2019) Functional genetic variation in the anti-Müllerian hormone pathway in women with polycystic ovary syndrome. J Clin Endocrinol Metab 104: 2855

Guleria C, Suri V, Kapoor R, Minz RW, Aggarwal R (2019) Human papillomavirus 16 infection alters the Toll-like receptors and downstream signaling cascade: a plausible early event in cervical squamous cell carcinoma development. Gynecol Oncol 155:151–160

Hedayat M, Netea MG, Rezaei N (2011) Targeting of Toll-like receptors: a decade of progress in combating infectious diseases. Lancet Infect Dis 11:702–712

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K et al (2002) Small antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 3:196–200

Hu P, Qiao F, Wang Y et al (2018) Clinical application of targeted next-generation sequencing on fetuses with congenital heart defects. Ultrasound Obstet Gynecol 52:205–211

Hudecova I, Sahota D, Heung MM (2014) Maternal plasma fetal DNA fractions in pregnancies with low and high risks for fetal chromosomal aneuploidies. PLoS One 9:e88484

Ibdah JA, Bennett MJ, Rinaldo P et al (1999) A fetal fatty-acid oxidation disorder as a cause of liver disease in pregnant women. N Engl J Med 340:1723

Iwabe T, Harada T, Tsudo T et al (2000) Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. J Clin Endocrinol Metab 85(2):824–829

Joura EA, Giuliano AR, Iversen OE et al (2015) Broad Spectrum HPV Vaccine Study. A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. N Engl J Med 372:711–723

Kanasaki K, Palmsten K, Sugimoto H et al (2008) Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. Nature 453:1117–1121

Kennedy S, Mardon H, Barlow D (1995) Familial endometriosis. J Assist Reprod Genet 12(1): 32–34

Klemmt PAB, Starzinski-Powitz A (2018) Molecular and cellular pathogenesis of endometriosis. Curr Womens Health Rev 14(2):106–116

Kurnit KC, Reid P, Moroney JW, Fleming GF (2020) Immune checkpoint inhibitors in women with gynecologic cancers: practical considerations. Gynecol Oncol S0090-8258(20):32325–32328. https://doi.org/10.1016/j.ygyno.2020.06.499

Lo YM (2013) Non-invasive prenatal testing using massively parallel sequencing of maternal plasma DNA: from molecular karyotyping to fetal whole-genome sequencing. Reprod Biomed Online 27:593–598

Lv W, Wei X, Guo R et al (2015) Noninvasive prenatal testing for Wilson disease by use of circulating single-molecule amplification and resequencing technology (cSMART). Clin Chem 61:172–181

Manegold C, Gravenor D, Woytowitz D, Mezger J, Hirsh V, Albert G et al (2008) Randomized phase II trial of a toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer. J Clin Oncol 26:3979–3986

Mao Q, Chin R, Xie W et al (2018) Advanced whole-genome sequencing and analysis of fetal genomes from amniotic fluid. Clin Chem 64:715–725

Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316:1628–1632

Maynard SE, Min JY, Merchan J et al (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 111:649–658

Natarajan SK, Ibdah JA (2018) Role of 3-hydroxy fatty acid-induced hepatic lipotoxicity in acute fatty liver of pregnancy. Int J Mol Sci 19:322

Olive DL, Schwartz LB (1993) Endometriosis. N Engl J Med 328(24):1759–1769

Ong FS, Lin JC, Das K et al (2013) Translational utility of next-generation sequencing. Genomics 102:137–139

Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12:252–264

Rasmussen M, Sunde L, Nielsen ML et al (2018) Targeted gene sequencing and whole-exome sequencing in autopsied fetuses with prenatally diagnosed kidney anomalies. Clin Genet 93: 860–869

Rosenfield RL, Ehrmann DA (2016) The Pathogenesis of Polycystic Ovary Syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr Rev 16(37):467

Salim Al-Gailani A, Angela Davis B (2014) Introduction to "Transforming pregnancy since 1900". Stud Hist Phil Biol Biomed Sci 47:229–232

Sata F, Yamada H, Suzuki K et al (2006) Functional maternal catechol-O-methyltransferase polymorphism and fetal growth restriction. Pharmacogenet Genomics 16:775–781

Sugimoto H, Hamano Y, Charytan D et al (2003) Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt1) induces proteinuria. J Biol Chem 278:12605–12608

Tiller GE, Kershberg HB, Goff J et al (2015) Women's views and the impact of noninvasive prenatal testing on procedures in a managed care setting. Prenat Diagn 35:428–433

Tran TT, Ahn J, Reau NS (2016) ACG clinical guideline: liver disease and pregnancy. Am J Gastroenterol 111:176

Tsao DS, Silas S, Landry BP et al (2019) A novel high-throughput molecular counting method with single base-pair resolution enables accurate single-gene NIPT. Sci Rep 9:14382

Tunbridge EM, Harrison PJ, Weinberger DR (2006) Catechol-o-methyltransferase, cognition, and psychosis: Val158Met and beyond. Biol Psychiatr 60:141–151

Vora NL, Hui L (2018) Next-generation sequencing and prenatal 'omics: advanced diagnostics and new insights into human development. Genet Med 20:791–799

Vuorela P, Helske S, Hornig C et al (2000) Amniotic fluid—soluble vascular endothelial growth factor receptor-1 in preeclampsia. Obstet Gynecol 95:353–357

Wellesley D, Dolk H, Boyd PA et al (2012) Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. Eur J Hum Genet 20:521–526

Wide L, Gamzell G (1960) Acta Endocrinol 35:261

Wong FC, Lo YM (2016) Prenatal diagnosis innovation: genome sequencing of maternal plasma. Annu Rev Med 67:419–432

Yang Z, Yamada J, Zhao Y et al (2002) Prospective screening for pediatric mitochondrial trifunctional protein defects in pregnancies complicated by liver disease. JAMA 288:2163

Yano T, Jimbo H, Yoshikawa H, Tsutsumi O, Taketani Y (1999) Molecular analysis of clonality in ovarian endometrial cysts. Gynecol Obstet Invest 47(Suppl 1):41–44

Yugawa T, Handa K, Narisawa-Saito M, Ohno SI, Fujita M, Kiyono T (2007) Regulation of Notch1 gene expression by p53 in epithelial cells. Mol Cell Biol 27:3732–3742

zur Hausen H (1976) Condylomata acuminate and human genital cancer. Can Res 36:530

Role of Macronutrients in Human Health and Disease

24

Paramjit S. Tappia and Anureet K. Shah

Abstract

A significantly large body of experimental, epidemiological, and clinical evidence has demonstrated a link between diet and adverse health outcomes. While bad nutritional practices and poor diets have a negative impact on health, specific dietary interventions are considered as important components of any nutrition-based approaches for disease treatment and prevention. Thus, there is not only an increase in public interest in human nutrition for health, but also scientific investigation into establishing dietary approaches that can be undertaken for the prevention and treatment has markedly increased. Accordingly, this article will briefly discuss recent advances in the investigation into the role of four major dietary components: carbohydrates, fats, protein hydrolysates, and bioactive peptides and prebiotics in human health and disease.

Keywords

Human nutrition · Carbohydrates · Dietary fats · Protein hydrolysates · Bioactive peptides · Prebiotics · Resistant starch · Health and disease

P. S. Tappia (✉)
Asper Clinical Research Institute, St. Boniface Hospital, Winnipeg, MB, Canada
e-mail: PTappia@sbrc.ca

A. K. Shah
Department of Kinesiology, Nutrition and Food Science, California State University, Los Angeles, CA, USA

R. C. Sobti, A. K. Ganju (eds.), *Biomedical Translational Research*,
https://doi.org/10.1007/978-981-16-8845-4_24

24.1 Introduction

The role of nutrition with respect to the amount and composition of the diet on disease risk is a component of nutritional sciences that is a highly impactful area of biomedical science (Schwingshackl et al. 2018). It is well established that nutrition plays an integral part throughout the life cycle starting from before birth and continues to affect us for our entire life depending on our diet selection. Consumption in excess or lack of specific nutrients can strongly impact our physiological functions. Imbalance of calories over a period of time can lead to physiological dysfunction. The indispensable role of nutrition in metabolism, health, and chronic disease makes it an integral part of biomedical sciences. With the advancement in nutritional research, there is a constant need to improve our understanding about varied dietary factors as causal agents or confounds (Davy and Davy 2019).

>The dietary guidelines in the USA recommend a healthy eating pattern that comprises a variety of fruits and vegetables, whole grains, fat-free or low-fat dairy, variety of proteins, and healthy oils. The guidelines also recommend to minimize calories from added sugars and saturated fats while limiting sodium intake. The Mediterranean diet and Dietary Approaches to Stop Hypertension (DASH) are also considered as recommended healthy eating patterns for disease prevention. Figure 24.1 depicts four important components of diet that can influence chronic diseases. Accordingly, this chapter briefly discusses recent advances in the investigation of the role of dietary carbohydrates, fats, protein hydrolysates, and bioactive peptides as well as prebiotics in human health and disease that may become important considerations in the formulation of dietary recommendations for disease treatment/prevention. In addition, we highlight the role of specific macronutrients in adverse health outcomes that warrant further investigation, and thus it is envisioned that this chapter will also prompt interest and stimulate research in the field of nutrition and human health.

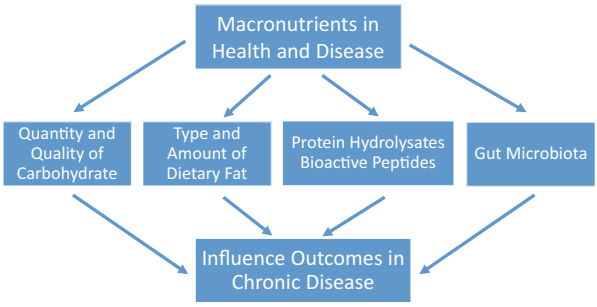


Fig. 24.1 Scheme depicting four important components of human nutrition that can influence chronic disease

24.2 Quantity and Quality of Carbohydrates in Chronic Disease

Carbohydrate is the only macronutrient with no established minimum dietary requirements. Interestingly, many populations with carbohydrate as their abundant energy source thrived equally well in comparison to the populations using it as their meagre energy source (Haglin 1991). The amount and type of carbohydrate necessary for optimal health and longevity is always a persistent question of prime concern and importance that has engaged researchers focusing on obesity, diabetes, cardiovascular disease, cancer, and early death (Ludwig et al. 2018). Several clinical trials have shown that low-carbohydrate diets in comparison to low-fat diet produce greater weight loss, which only provides short-term benefit because such diets are associated with poor long-term compliance (Mansoor et al. 2016; Mancini et al. 2016; Bueno et al. 2013). Also, some recent studies have reported non-significant advantage for a healthy low carbohydrate versus low fat as long as both groups minimized sugar, refined grains, and processed food in general (Gardner et al. 2018).

Some preliminary evidence from research studies suggest the metabolic advantage of low carbohydrate and low glycemic index diets in both type 1 and type 2 diabetes as observed through improved glycemic control and lower triglycerides. A continual research study providing long-term data on safety and efficacy is required to further substantiate its effect (Feinman et al. 2015; Lennerz et al. 2018). Some of the long-term large cohort studies in the US have shown the impact of total carbohydrate on higher mortality rates where type of dietary fat was used as a modifiable risk factor (Wang et al. 2016). In accordance, substitution of saturated fat with low glycemic index and high glycemic index carbohydrates resulted in lower and higher risk of myocardial infarction respectively (Jakobsen et al. 2010). These studies suggest that quality of carbohydrates may have a greater effect on health rather than the quantity. Many controversies still exist, despite recent developments and research about the metabolic effects of carbohydrate in areas of wide consensus. Ludwig et al. (2018) have discussed the flaws in the data from long-term observational studies, which may be confounded and have methodological concerns. Furthermore, most randomized control trials (RCTs) are short in duration, lack blinding, do not control for treatment intensity between groups and have poor compliance. The resolution of these studies will require the progressive research design involving nutrigenomics, where understanding of specific gene types in certain individuals may establish the susceptibility/risk to certain chronic disease. It is interesting to note that the presence of high or low copy number of the salivary amylase gene, AMY 1, which in turn affects amylase protein concentration in saliva, has been reported (Falchi et al. 2014; Rukh et al. 2017), suggesting that genetic makeup is an important factor in determining early digestion capacity. Future research models should be designed to understand the presence and absence of many such genes that may play a role in carbohydrate metabolism and thereby influence disease outcomes. Indeed, nutrigenomics continues to be an important area of nutritional science investigation in relation to human health.

Added sugars are sugar carbohydrates that are added to food and beverages during processing or manufacturing. This type of sugar is chemically

indistinguishable from naturally occurring sugars and include brown sugar, corn syrup, dextrose, fructose, glucose, sucrose, and raw sugar. It should be noted that the leading sources of added sugars in the US diet are sugar-sweetened beverages, grain-based desserts such as cakes and cookies, and dairy desserts such as ice cream. The US dietary guidelines have recommended that intake of added sugars should be $<10\%$ of the total daily calories. In this regard, in the USA, between 2003 and 2010, 14% of the total caloric intake in people aged 6 years of age and older was from added sugars (Drewnowski and Rehm 2014); in 2005–2010, the average % of total daily calories from added sugar was 13% in both men and women aged 20 years and older (Ervin et al. 2012). In 2005–2008, the average % of total daily calories from added sugar was 16% for both boys and girls aged between 2 and 19 years of age (Park et al. 2016). The contribution and mechanisms of action of these different added sugars to several chronic diseases remain a topic for debate. According to the WHO (World Health Organization), overconsumption of added sugars specifically sugar-sweetened drinks has led to the obesity epidemic (WHO 2015). Several high-quality observational studies showed the direct link of overconsumption of added sugars with energy intake and obesity (Vartanian et al. 2007; Malik et al. 2013). Apart from body weight, several randomized controlled trials indicated direct link of added sugars with total cholesterol level, triglycerides, blood pressure, and cardiovascular disorders (Malik Vasanti and Hu 2015; Te Morenga et al. 2014). Most of the above-mentioned studies have their limitations with respect to lack of control group, which was confounded by unintended weight loss. The relative contribution of added sugars in the occurrence of several chronic diseases remains unknown and requires extensive research involving more sophisticated study designs.

24.3 Dietary Fat and Cardiovascular Health

In addition to being a source of energy, fatty acids also exhibit a wide spectrum of biological activities. They are the important constituents of all cell membranes imparting structural function, precursors for the synthesis of hormones and bile salts, act as a store house of energy and facilitates the absorption of fat-soluble vitamins. In spite of their crucial functions, recommendations are put forward to minimize the intake of total fat, replace saturated fatty acids (SFAs) with unsaturated fatty acids and possibly avoid the intake of trans fat. These above-mentioned recommendations are part of a primary nutrition interventional approach to prevent the occurrence of coronary heart disease (CHD) and cardiovascular disease (CVD) (Aranceta and Pérez-Rodrigo 2012).

Serum cholesterol levels have been linked to varied outcomes in different cohorts. In this regard, in the 1980s, the “lipid theory” came into existence, where SFAs as a percentage of calories were considered the most powerful lifestyle predictor of heart disease. The lipid theory propagated that diet rich in SFAs would increase the total serum cholesterol and deposition of arterial plaques, which will eventually lead to CVD.

Evidence from other studies relating the effect of SFA consumption and other risk predictors such as insulin resistance and diabetes has been inconsistent with no clear outcomes. In an intervention review of the Cochrane Collaboration, the effect of replacing SFAs with monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), carbohydrates, and proteins as an outcome on mortality and cardiovascular morbidity was examined (Hooper et al. 2015). Several RCTs demonstrated the positive impact of replacing SFAs with PUFAs, but with relatively no positive impact when replaced with carbohydrates and proteins. Some large prospective cohort studies have reported a direct relationship between dietary intake of SFAs and incidence of CVD, however several other RCTs have demonstrated no direct relationship. On the other hand, some but not all clinical trials have confirmed the reduction in cardiovascular outcomes after consumption of omega-3 PUFA (Burr et al. 2003). There are a number of factors that could explain the inconsistencies in the outcomes of the omega-3 PUFA RCTs and of meta-analyses. For example, patient demographic diversity and disease conditions, differences in drug regimens, mixed end points, as well as duration of the intervention could be important aspects that would contribute to such inconsistencies. In addition, variability in the doses, purity and formulations of omega-3 PUFAs (eicosapentaenoic acid (EPA): docosahexaenoic acid (DHA) ratios), differences in the use of placebo, estimation of the omega-3 PUFA plasma levels from dietary intake or actual measurements of omega-3 levels at pre- and post-treatment stages as well as differential uptake of omega-3 PUFAs could also be key determinants of trial outcomes as well as patient compliance (Shaikh and Tappia 2015; Xu et al. 2014).

Despite the inconsistencies of the data on omega-3 PUFAs on cardiovascular health outcomes, the American Heart Association recommends 1 g of EPA/DHA per day for patients with CHD (Kris-Etherton et al. 2003). However, higher doses are required to improve lipid profiles in hyper-triglyceridemic patients, as well as to improve systolic left ventricle function in chronic heart failure, stabilize atherosclerotic plaques, and to reduce arterial stiffening and atherosclerosis. While public awareness on the cardiovascular health benefits of omega-3 fatty acids have progressively increased over the last few years, there is still a need for well-designed, randomized controlled trials in different populations with respect to etiology of CVD. Furthermore, future trials also need to take age, sex, and ethnicity into consideration in the response to dietary or supplemental omega-3 PUFAs (Tappia and Shah 2020). While this section has focused on marine sources of omega-3 PUFAs, the clinical efficacy of plant sources of omega-3 PUFAs such as alpha-linolenic acid (Parikh et al. 2019; Rodriguez-Leyva et al. 2010; Bassett et al. 2009) either alone or in combination with marine sources of omega-3 PUFAs warrants further investigation. Similarly, krill oil could also emerge as an important source of EPA and DHA (bound mostly to phospholipid), but requires extensive further testing.

Although most of the ardioprotective effects of omega-6 is attributed towards the usage of linoleic acid (Jakobsen et al. 2009), a recent meta-analysis failed to show any promising effect of omega-6 and decrease in coronary events (Chowdhury et al. 2014). The general consensus about the favorable effects of the Mediterranean diet

for CHD prevention is quite prevalent, however different observational studies showed discordant effects of MUFAs on CVD (Jakobsen et al. 2009). Trans fatty acids (TFAs) in comparison to cis isomers not only raised LDL-cholesterol levels but lowered HDL-cholesterol levels. TFA seem to increase the plasma activity of cholesteryl ester transfer protein (CETP), which is probably the contributing factor in lowering HDL and decreasing LDL (van Tol et al. 1995).

The important criticism for discordant results from these studies is that fats never occur alone in the diet but always co-exist in several foodstuffs. To understand the metabolic effect of dietary fat, it is best to understand the role of certain genes (APO-E), which make certain individuals more prone than others. Nevertheless, the dietary recommendations to take overall diet into account rather than the simple pure ingredient would be the ideal research target for futuristic approach (Fattore and Massa 2018).

24.4 Protein Hydrolysates for Human Health Benefits

The need to reduce negative side effects (nausea, vomiting, dry cough) of some of the currently prescribed antihypertensive therapy has spurred research into alternative natural sources of effective compounds such as food protein-derived peptides. Recently, an emerging recent interest for nutrition and human health has been protein hydrolysates and food-derived biopeptides. Bioactive peptides are specific protein fragments, which are inactive within the parent polypeptide. These biopeptides can exert a variety of biological and physiological actions, including antioxidant effects, blood pressure-lowering properties, and antithrombotic, hypoglycemic effects as well as improving blood lipid profile that is dependent on the amino acid sequence and the specificity of the hydrolyzing enzyme employed (Korhonen and Pihlanto 2003). Protein hydrolysates are produced by enzymatic hydrolysis of whole protein sources by using appropriate proteolytic enzymes under controlled conditions, followed by post hydrolysis processing to isolate desired and potent bioactive peptides from a complex mixture of active and inactive peptides. Although the antioxidant and anti-inflammatory actions of several different food-derived proteins including hydrolysates and their fractions have been reported (Piotrowicz et al. 2020; Suarez-Jimenez et al. 2012; Bueno-Gavila et al. 2019; Zamora-Sillero et al. 2018; Leee et al. 2017; Nongonierma and FitzGerald 2015), several other studies have also reported antihypertensive properties of protein isolates and hydrolysate isolated from different sources including flaxseed, hempseed, kidney beans, pistachio, and corn (Doyen et al. 2014; Girgih et al. 2014a; Mundi and Aluko 2014; Li et al. 2014; Huang et al. 2011). In addition, we have earlier shown thermolysin-derived bioactive peptides from pea protein hydrolysates that contained <3 kDa peptides reduced blood pressure in hypertensive rats and human subjects, likely via effects on the renal angiotensin system (Li et al. 2011).

It should be mentioned that angiotensin converting enzyme (ACE) is responsible for the conversion of angiotensin I, an inactive decapeptide to angiotensin II, a very

potent vasoconstrictor that also enhances sodium (fluid) retention and is thus a key target for treatment of hypertension. As a consequence of these observations on blood pressure, hypertension treatment other than ACE inhibition has been suggested (Norris and FitzGerald 2013). Such alternative antihypertensive compounds target renin, and bioactive peptides from plant proteins have been shown to have renin- and ACE-inhibitory properties (Girgih et al. 2014a, b). Overall, the literature supports the use of bioactive peptides as part of the formulation of functional foods and nutraceuticals for the treatment and/or prevention of hypertension in high risk populations.

24.5 Diet and Gut Microbiota

Although the term "gut health" remains to be clearly defined (Bischoff 2011), a number of criteria for healthy gastrointestinal (GI) system have been used to form the basis for a positive and objective definition of gut health (Bischoff 2011). These include the effective digestion and absorption of food (including regular bowel movement, no abdominal pain, and normal stool consistency), absence of GI illness (including no inflammatory bowel disease, no other inflammatory state, no colorectal or other GI cancers), and normal and stable intestinal microbiota (including no bacterial overgrowth, normal composition, no GI infections). The significance of intestinal microbiota in gut health has long been recognized and microbial compositional changes have been demonstrated to occur with antibiotic therapy, enteric infections, and aging (Raskin 2008; Claesson et al. 2011, 2012; Biagi et al. 2012; Comparato et al. 2007). In healthy adults the gut microbiome contains 10^{11} to 10^{12} bacteria per gram of stool. Firmicutes (e.g., Clostridum cluster IV and XIVa and Bifidobacteria) are the primary component representing 50–80% of the bowl microbiome with Bacteroidetes being the next most prevalent group at 10–30% (Biagi et al. 2012).

The role of the intestinal microbiota in human health is gaining more attention since clear changes in the composition of the intestinal bacteria or environment are seen in patients with inflammatory bowel disease, allergy, autoimmune disease, and some lifestyle-related illnesses (Kataoka 2016). Recent studies have suggested that the intestinal microbiome plays an important role in modulating risk of several other chronic diseases, including inflammatory bowel disease, obesity, type 2 diabetes, cardiovascular disease, and cancer as well as being implicated in neurodevelopmental conditions such as autism spectrum disorder (Barko et al. 2018) and behavior (Cresci and Bawden 2015). It is now also understood that diet plays a significant role in shaping the microbiome, with experiments showing that dietary alterations can induce large, temporary microbial shifts within 24 h. Given this association, there may be significant therapeutic utility in altering microbial composition through diet (Singh et al. 2017). A healthy gut environment is regulated by the exquisite balance of intestinal microbiota, metabolites, and the host's immune system. Imbalance of these factors in genetically susceptible persons may promote a disease state. Manipulation of the intestinal microbiota with prebiotics, which can

selectively stimulate growth of beneficial bacteria, might help to maintain a healthy intestinal environment or improve diseased one (Kataoka 2016). Accordingly, new technologies have led the science world to wonder about the impact that the gut microbiota may have on human health and disease (Cresci and Bawden 2015).

Aging is a natural process of organismal decay that underpins the development of myriad diseases and disorders. In fact, microbiome dysbiosis is now considered as another characteristic biomarker of aging (Bana and Cabreiro 2019). However, elderly and younger populations do show differences in gut microbe composition (Maynard and Weinkove 2018). Many countries are facing aging populations, with those over 65 years of age likely to represent the largest population over the next 10–20 years. Living longer often comes with poorer health and, in particular, a decline in the immune function (Clements and Carding 2018). Gut dysbiosis can trigger the innate immune response and chronic low-grade inflammation, leading to many age-related degenerative pathologies and unhealthy aging (Kim and Jazwinski 2018). Biagi et al. (2012) eloquently reviewed how aging of humans is linked with gut microbiome changes and how this may be linked to diet in the elderly. The microbiome of the large bowel enhances the host's metabolic ability by hydrolyzing complex plant polysaccharides and starches that are poorly digested by the human digestive tract. This hydrolysis is primarily done by Firmicutes in the large bowel and leads to the production of short chain fatty acids (SCFAs) that provide an absorbable source of energy for the host. Furthermore, SCFAs are considered to contribute to optimal GI function (Topping and Clifton 2001; Kendall et al. 2004). Butyrate is particularly important as it maintains normal cell population and gut health (Topping and Clifton 2001; Kendall et al. 2004). The gut microbiome also produces essential vitamins (e.g., B12) that are required by the host (Biagi et al. 2012).

Stewart et al. (2010) reported that the average fiber intake in the USA (similar to Canadian data) is 13–18 g/day, which is half the recommended intake of 25 g/day for women and 38 g/day for men. It is pointed out the health benefits of higher dietary fiber intakes are well established (Fayet-Moore et al. 2018a, b). A recently conducted systematic review and meta-analysis have demonstrated that the benefits of dietary fiber intakes on total mortality, incidences of cardiovascular disease, type 2 diabetes, and colorectal cancer are likely to be dose-response dependent (Reynolds et al. 2019). In addition, it was reported that for every 8 g increase of dietary fiber consumed per day, the total number of deaths, incidence of CHD, type 2 diabetes, and colorectal cancer would be reduced by 2–19% with a further benefit achieved by intakes of fiber in the diet greater than 30 g (Reynolds et al. 2019). In view of the importance and relationship of gut microflora to human health, research on dietary fibers has also markedly increased over the last few years. Dietary guidelines recommend a daily dietary resistance starch (RS) intake of 15–20 g, however; Canadians consume between 3 and 8 g RS/day. Clearly, RS consumption among Canadians is low. This has been partially attributed to the variability of RS content in common foods and a general lack of commercially available RS-enriched foods and nutraceutical products.

A gradual immunosenesescence (age-dependant deterioration of the immune system) occurs as humans age (Biagi et al. 2012). The decreased gut inflammatory function results in a shift in the balance between protective symbionts (microbes that are helpful) and pathobionts (microbes that are potential pathogens) in favor of the latter (Sansonetti 2011). This age-related increase in facultative anaerobes (pathobionts) and decrease in Firmicutes and Bacteroidetes (symbionts) in the elderly are thought to be linked to a variety of factors including diet. Figure 24.2 shows that at birth the human gut gradually becomes colonized with microbes and by approximately 10 years of age, gut microbiome is stable that persists to approximately 60 years of age when a gradual shift in the microbiome occurs resulting in a reduction in the Firmicutes/Bacteroidetes level and an increase in facultative anaerobes belonging to the Proteobacteria. These changes in microbiome in the elderly are linked to compromised gut health and prebiotics (e.g., lactose and inunlin) may reverse this age-related microbiome shift (Toward et al. 2012). There is evidence of increased diverticular disease in the elderly that is associated with localized inflammation, which is exacerbated by a low-fiber diet (Raskin 2008). Constipation is also common in the elderly and is caused by several factors including inactivity and inappropriate diet (Leung and Rao 2009; Spinzi et al. 2009).

It has been suggested that maintenance of a balanced, healthy gut microbiome during the ageing process may delay or even prevent the inflamm-aging process that occurs in the elderly (Biagi et al. 2012). Indeed, in Ireland the ELDERMET consortium was established to assess the intestinal microbiota in the elderly Irish population to determine its role in maintaining gut health. A small UK study reported malnutrition in 10% of people (mean age 79 years) living in sheltered accommodation (Harris et al. 2008). Studying the gut microbiome is difficult as the majority of human gut bacteria cannot be grown in the laboratory (Duncan et al. 2007; Eckburg et al. 2005). However, newly developed genetic methods such as pyrosequencing provide a powerful means to study how the gut ecosystem changes (Schellenberg et al. 2009; Chang et al. 2011; Rea et al. 2011).

Protection of the gut from infectious agents can be achieved using antibiotic therapy once infection occurs, but the potential for gut organisms to develop resistance to these antibiotics has stimulated research to find alternative ways to protect humans from infection. One such approach has been the use of prebiotics. Use of prebiotics such as indigestible starch has been suggested as a valuable approach in the elderly (Stewart et al. 2010; Haub et al. 2010; Jenkins et al. 1998; O'Keefe 2010). Resistant starch can be derived from a variety of food sources. RS1 is starch that is inaccessible to digestive enzymes because it is embedded in a matrix. RS2 is untreated granules of starch that may be derived from potato, green bananas, or high-amylose corn starches. RS3 is cooked starch that is non-granule, but re-associates and recrystallizes when it cools. Starches that are structurally modified are called RS4. These RS compounds are important as they are fermented in the colon by bacteria such as Bifidobacteria to form short chain fatty acids (SCFA). These SCFAs (most commonly in the form of acetate, propionate, butyrate) in the colon account for about 10% of the total energy intake in humans (Jenkins et al. 1998). Some studies (Lee 2008; Kleessen et al. 1997) indicate that the use of elderly

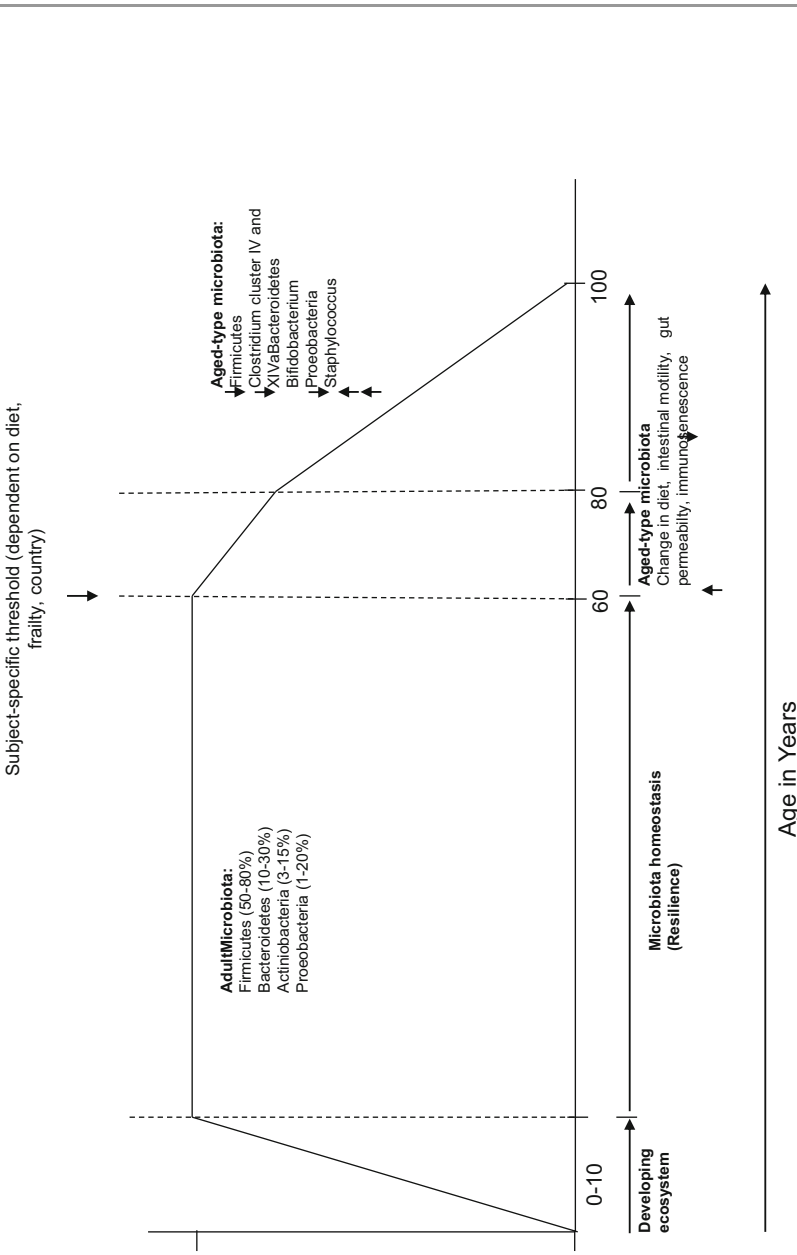


Fig. 24.2 Dynamics and age-dependent changes of the gut microbial. At birth, the human gut gradually becomes colonized with microbes. At approximately 10 years of age, gut microbiome is stable, which remains until about age 55–60 years of age when a gradual shift in the microbiome occurs toward a reduction in the Firmicutes/Bacteroidetes level and an increase in facultative anaerobes belonging to the Proteobacteria (Adapted from Biagi et al. 2012)

elemental diets for nutrition does not have adequate indigestible carbohydrate and that this predisposes the gut to infection with *C. difficile*. Kleessen et al. (1997) reported that inulin as a prebiotic improved constipation in 9/10 subjects. Furthermore, it has been reported that the prebiotic oligofructose significantly reduced diarrhea recurrence in patients with *C. difficile* associated disease and that this correlated with an increase in the level of Bifidobacteria (a member of the Firmicutes) in the gut of these patients (Lewis et al. 2005). An RS2 preparation derived from potatoes has been reported to improve intestinal health and favourable shifts in gut microbial populations as well as reduction of scours in baby pigs (Bhandari et al. 2009). In this regard, we have earlier demonstrated that this RS2 from potato starch meets the criteria of a prebiotic and can stimulate an increased abundance of endogenous Bifidobacteria in both the elderly as well as mid-aged populations, and was found to eliminate the dysbiosis of gut Proteobacteria observed in the elderly (Alfa et al. 2018a). Interestingly, we also observed that it was able to reduce insulin resistance, a major risk factor for type 2 diabetes, in the elderly (Alfa et al. 2018b). From the aforementioned it is evident that manipulation of the gut microflora through dietary interventions has a significant role to play in age-related chronic disease and human health and thus represents a very important and potentially fruitful area of research in nutrition and human health.

24.6 Conclusions

It is clear that nutrition plays an important role in human health and disease. Although benefit to human health may not be due to a single nutrient, a balanced and varied diet of food items that can provide different benefits may prove to be key to prevention of chronic diseases. In general, nutrients exhibit a diverse range of properties including anti-oxidant effects, anti-inflammatory actions, modification of signal transduction mechanisms, as well as metabolic, molecular, and membrane actions. While the quality of carbohydrates have been suggested to exert adverse health effects more than the quantity, their contribution and mechanisms of actions as well as that of added sugars to several chronic diseases remains to be examined. Although the data from RCTs with omega-3 PUFAs on cardiovascular health outcomes are inconsistent, the American Heart Association recommends 1 g of EPA/DHA per day for patients with CHD. Alternate plant sources of omega-3 fatty acids have also gained a lot of interest particularly in relation to CVD. In view of the relationship between the composition of the gut microflora and different pathophysiological conditions including obesity, diabetes, and CVD, the field of probiotics as well as prebiotics has also exponentially increased. In view of the potential value for human health and being natural food-derived products, protein hydrolysates, and biopeptides may be useful as ingredients in functional foods and as nutraceuticals for treatment/prevention of chronic diseases including hypertension and cancer. In this brief review, we have provided information clearly; further investigation and advancement in understanding the essential role of nutrition in the treatment and/or prevention of a variety of human chronic diseases is warranted.

Acknowledgements Infrastructural support was provided by the St. Boniface Hospital Albrechtsen Research Centre.

References

Alfa MJ, Strang D, Tappia PS et al (2018a) A randomized trial to determine the impact of a digestion resistant starch composition on the gut microbiome in older and mid-age adults. Clin Nutr 37:797–807

Alfa MJ, Strang D, Tappia PS et al (2018b) A randomized placebo controlled clinical trial to determine the impact of digestion resistant starch *MSPrebiotic*® on glucose, insulin, and insulin resistance in elderly and mid-age adults. Front Med 4:260

Aranceta J, Pérez-Rodrigo C (2012) Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: a systematic review. Br J Nutr 107(Suppl 2):S8–S22

Bana B, Cabreiro F (2019) The microbiome and aging. Annu Rev Genet 53:239–261

Barko PC, McMichael MA, Swanson KS, Williams DA (2018) The gastrointestinal microbiome: a review. J Vet Intern Med 32:9–25

Bassett CM, Rodriguez-Leyva D, Pierce GN (2009) Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. Appl Physiol Nutr Metab 34:965–974

Bhandari SK, Nyachoti CM, Krause DO (2009) Raw potato starch in weaned pig diet and its influence on postweaning scours and the molecular microbial ecology of the digestive tract. J Anim Sci 87:984–993

Biagi E, Candela M, Fairweather-Tait S et al (2012) Ageing of the human metaorganism: the microbial counterpart. Age 34:247–267

Bischoff SC (2011) "Gut health": a new objective in medicine? BMC Med 9:24

Bueno NB, de Melo IS, de Oliveira SL, da Rocha Ataide T (2013) Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: a meta-analysis of randomised controlled trials. Br J Nutr 110:1178–1187

Bueno-Gavila E, Abellan A, Giron-Rodriguez F et al (2019) Bioactivity of hydrolysates obtained from bovine casein using artichoke (Cynara scolymus L.). J Dairy Sci 102:10711–10723

Burr ML, Ashfield-Watt PA, Dunstan FD et al (2003) Lack of benefit of dietary advice to men with angina: results of a controlled trial. Eur J Clin Nutr 57:193–200

Chang JY, Shin SM, Chun J et al (2011) Pyrosequencing-based molecular monitoring of the intestinal bacterial colonization in preterm infants. J Pediatr Gastroenterol Nutr 53:512–519

Chowdhury R, Warnakula S, Kunutsor S et al (2014) Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. Ann Intern Med 160:398–406

Claesson MJ, Cusack S, O'Sullivan O et al (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci 108:4586–4591

Claesson MJ, Jeffery IB, Conde S et al (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488:178–184

Clements SJ, Carding SR (2018) Diet, the intestinal microbiota, and immune health in aging. Crit Rev Food Sci Nutr 58:651–661

Comparato G, Pilotto A, Franzé A et al (2007) Diverticular disease in the elderly. Dig Dis 25:151–159

Cresci GA, Bawden E (2015) Gut microbiome: what we do and don't know. Nutr Clin Pract 30:734–746

Davy KP, Davy BM (2019) Advances in nutrition science and integrative physiology: insights from controlled feeding studies. Front Physiol 10:1341

Doyen A, Udenigwe CC, Mitchell PL et al (2014) Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. Food Chem 145:66–76

Drewnowski A, Rehm CD (2014) Consumption of added sugars among US children and adults by food purchase location and food source. Am J Clin Nutr 100:901–907
Duncan SH, Louis P, Flint HJ (2007) Cultivable bacterial diversity from the human colon. Lett Appl Microbiol 44:343–350
Eckburg PB, Bik EM, Bernstein CN et al (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638
Ervin RB, Kit BK, Carroll MD, Ogden CL (2012) Consumption of added sugar among US children and adolescents, 2005-2008. NCHS Data Brief 87:1–8
Falchi M, El-Sayed Moustafa JS, Takousis P et al (2014) Low copy number of the salivary amylase gene predisposes to obesity. Nat Genet 46:492–497
Fattore E, Massa E (2018) Dietary fats and cardiovascular health: a summary of the scientific evidence and current debate. Int J Food Sci Nutr 69:916–927
Fayet-Moore F, Cassettari T, Tuck K et al (2018a) Dietary fibre intake in Australia. Paper I: associations with demographic, socio-economic, and anthropometric factors. Nutrients 10:599
Fayet-Moore F, Cassettari T, Tuck K et al (2018b) Dietary fibre intake in Australia. Paper II: comparative examination of food sources of fibre among high and low fibre consumers. Nutrients 10:1223
Feinman RD, Pogozelski WK, Astrup A et al (2015) Dietary carbohydrate restriction as the first approach in diabetes management: critical review and evidence base. Nutrition 31:1–13
Gardner CD, Trepanowski JF, Del Gobbo LC et al (2018) Effect of low-fat vs low-carbohydrate diet on 12-month weight loss in overweight adults and the association with genotype pattern or insulin secretion: the DIETFITS randomized clinical trial. JAMA 319:667–679
Girgih AT, He R, Alashi AM et al (2014a) Preventive and treatment effects of hemp seed (Cannabis sativa L.) meal protein hydrolysate against blood pressure in spontaneously hypertensive rats. Eur J Nutr 53:1237–1246
Girgih AT, He R, Malomo SA, Aluko RE (2014b) Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. J Funct Foods 6:384–394
Haglin L (1991) Nutrient intake among Saami people today compared with an old, traditional Saami diet. Arctic Med Res (Suppl):741–746
Harris DG, Davies C, Ward H et al (2008) An observational study of screening for malnutrition in elderly people living in sheltered accommodation. J Hum Nutr Diet 21:3–9
Haub ME, Hubach KL, Al-Tamini EK et al (2010) Different types of resistant starch elicit different glucose responses in humans. J Nutr Metab 2010:230501
Hooper L, Martin N, Abdelhamid A, Davey Smith G (2015) Reduction in saturated fat intake for cardiovascular disease. Cochrane Database Syst Rev 6:CD011737
Huang WH, Sun J, He H et al (2011) Antihypertensive effect of corn peptides, produced by a continuous production in enzymatic membrane reactor, in spontaneously hypertensive rats. Food Chem 128:968–973
Jakobsen MU, O'Reilly EJ, Heitmann BL et al (2009) Major types of dietary fat and risk of coronary heart disease: a pooled analysis of 11 cohort studies. Am J Clin Nutr 89:1425–1432
Jakobsen MU, Dethlefsen C, Joensen AM et al (2010) Intake of carbohydrates compared with intake of saturated fatty acids and risk of myocardial infarction: importance of the glycemic index. Am J Clin Nutr 91:1764–1768
Jenkins D, Vuksan V, Kendall C et al (1998) Physiological effects of resistant starches on fecal bulk, short chain fatty acids, blood lipids and glycemic index. J Am Coll Nutr 17:609–616
Kataoka K (2016) The intestinal microbiota and its role in human health and disease. J Med Invest 63:27–37
Kendall CW, Emam A, Augustin LS et al (2004) Resistant starches and health. J AOAC Int 87:769–774
Kim S, Jazwinski SM (2018) The gut microbiota and healthy aging: a mini-review. Gerontology 64: S13–S20

Kleessen B, Sycura B, Zunft HJ et al (1997) Effects of inulin and lactose on fecal microflora, microbial activity and bowel habits in elderly constipated persons. Am J Clin Nutr 65:1397–1402

Korhonen H, Pihlanto A (2003) Food-derived bioactive peptides—opportunities for designing future foods. Curr Pharm Des 9:1297–1308

Kris-Etherton PM, Harris WS, Appel LJ (2003) Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. Arterioscler Thromb Vasc Biol 23:151–152

Lee WJ (2008) Bacterial-modulated signaling pathways in gut homeostasis. Sci Signal 1:pe24

Leee D, Bamdad F, Khey K, Sunwoo HH (2017) Antioxidant and anti-inflammatory properties of chicken egg vitelline membrane hydrolysates. Poult Sci 96:3510–3516

Lennerz BS, Barton A, Bernstein RK et al (2018) Management of type 1 diabetes with a very low-carbohydrate diet. Pediatrics 141:e20173349

Leung FW, Rao SS (2009) Fecal incontinence in the elderly. Gastroenterol Clin North Am 38:503–511

Lewis S, Burmeister S, Brazier J (2005) Effect of the prebiotic oligofructose on relapse of Clostridium difficile-associated diarrhea: a randomized, controlled study. Clin Gastroenterol Hepatol 3:442–448

Li H, Prairie N, Udenigwe CC et al (2011) Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. J Agric Food Chem 59:9854–9860

Li P, Jia J, Fang M et al (2014) In vitro and in vivo ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. Process Biochem 49:898–904

Ludwig DS, Hu FB, Tappy L, Brand-Miller J (2018) Dietary carbohydrates: role of quality and quantity in chronic disease. BMJ 361:k2340

Malik Vasanti S, Hu Frank B (2015) Fructose and cardiometabolic health. J Am Coll Cardiol 66: 1615–1624

Malik VS, Pan A, Willett WC, Hu FB (2013) Sugar-sweetened beverages and weight gain in children and adults: a systematic review and meta-analysis. Am J Clin Nutr 98:1084–1102

Mancini JG, Filion KB, Atallah R, Eisenberg MJ (2016) Systematic review of the Mediterranean diet for long-term weight loss. Am J Med 129:407–415

Mansoor N, Vinknes KJ, Veierod MB, Retterstol K (2016) Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors: a meta-analysis of randomised controlled trials. Br J Nutr 115:466–479

Maynard C, Weinkove D (2018) The gut microbiota and aging. Subcell Biochem 90:351–371

Mundi S, Aluko RE (2014) Inhibitory properties of kidney bean protein hydrolysate and its membrane fractions against renin, angiotensin converting enzyme, and free radicals. Austin J Nutr Food Sci 2:1–11

Nongonierma AB, FitzGerald R (2015) Bioactive properties of milk proteins in humans: a review. Peptides 73:20–34

Norris R, FitzGerald R (2013) Antihypertensive peptides from food proteins. In: Hernandez-Ledesma B, Hsieh C (eds) Bioactive food peptides in health and disease. INTECH Publishers, Rijeka, pp 45–72

O'Keefe SJD (2010) Tube feeding, the microbiota, and Clostridium difficile infection. World J Gastroenterol 16:139–142

Parikh M, Raj P, Austria JA et al (2019) Dietary flaxseed protects against ventricular arrhythmias and left ventricular dilation after a myocardial infarction. J Nutr Biochem 71:63–71

Park S, Thompson F, Pan L et al (2016) Sociodemographic and behavioral factors associated with added sugars intake among US adults. J Acad Nutr Diet 116:1589–1598

Piotrowicz IBB, Garces-Rimon M, Moreno-Fernandez S et al (2020) Antioxidant, angiotensin-converting enzyme inhibitory properties and blood-pressure-lowering effect of rice bran protein hydrolysates. Foods 9:812

Raskin MJ (2008) History, incidence, and epidemiology of diverticulosis. J Clin Gastroenterol 42: 1125–1127

Rea MC, Dobson A, O'Sullivan O et al (2011) Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. Proc Natl Acad Sci 108:4639–4644
Reynolds A, Mann J, Cummings J et al (2019) Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. Lancet 393:434–445
Rodriguez-Leyva D, Dupasquier CM, McCullough R, Pierce GN (2010) The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid. Can J Cardiol 26:489–496
Rukh G, Ericson U, Andersson-Assarsson J et al (2017) Dietary starch intake modifies the relation between copy number variation in the salivary amylase gene and BMI. Am J Clin Nutr 106:256–262
Sansonetti PJ (2011) To be or not to be a pathogen: that is the mucosally relevant question. Mucosal Immunol 4:8–14
Schellenberg J, Links MG, Hill JE et al (2009) Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. Appl Environ Microbiol 75:2889–2898
Schwingshackl L, Bogensberger B, Hoffmann G (2018) Diet quality as assessed by the healthy eating index, alternate healthy eating index, dietary approaches to stop hypertension score, and health outcomes: an updated systematic review and meta-analysis of cohort studies. J Acad Nutr Diet 118:74–100
Shaikh NA, Tappia PS (2015) Why are there inconsistencies in the outcomes of some omega-3 fatty acid trials for the management of CVD? Clin Lipidol 10:27–42
Singh RK, Chang H-W, Yan D et al (2017) Influence of diet on the gut microbiome and implications for human health. J Transl Med 15:73
Spinzi G, Amato A, Imperiali G et al (2009) Constipation in the elderly: management strategies. Drugs Aging 26:469–474
Stewart ML, Nikhanz SD, Timm D-A et al (2010) Evaluation of the effect of four fibers on laxation, gastrointestinal tolerance and serum markers in healthy humans. Ann Nutr Metab 56:91–98
Suarez-Jimenez G-M, Burgos-Hernandez A, Ezquerra-Brauer J-M (2012) Bioactive peptides and depsipeptides with anticancer potential: sources from marine animals. Mar Drugs 10:963–986
Tappia PS, Shah AK (2020) Sex differences in response to fatty acids in cardiovascular health and disease. In: Sex Differences in Heart Disease, Ostadal B, Dhalla NS (eds) Springer Nature Switzerland AG, 191–202
Te Morenga LA, Howatson AJ, Jones RM, Mann J (2014) Dietary sugars and cardiometabolic risk: systematic review and meta-analyses of randomized controlled trials of the effects on blood pressure and lipids. Am J Clin Nutr 100:65–79
Topping DL, Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81:1031–1064
Toward RE, Montandon SL, Walton GE et al (2012) Effect of prebiotics on the human gut microbiota of elderly persons. Gut Microbes 3:57–60
van Tol A, Zock PL, van Gent T et al (1995) Dietary trans fatty acids increase serum cholesterylester transfer protein activity in man. Atherosclerosis 115:129–134
Vartanian LR, Schwartz MB, Brownell KD (2007) Effects of soft drink consumption on nutrition and health: a systematic review and meta-analysis. Am J Public Health 97:667–675
Wang DD, Li Y, Chiuve SE et al (2016) Association of specific dietary fats with total and cause-specific mortality. JAMA Intern Med 176:1134–1145
WHO (2015) Sugars intake for adults and children: guideline. WHO
Xu YJ, Gregor T, Dhalla NS, Tappia PS (2014) Is the jury still out on the benefits of fish, seal and flax oils in cardiovascular disease? Ann Nutr Disord Ther 1:3
Zamora-Sillero J, Gharsallaoui A, Prentice C (2018) Peptides from fish by-product protein hydrolysates and its functional properties: an overview. Mar Biotechnol 20:118–130