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The Role of Glycosylation in Health and Disease

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Preface

Glycobiology is an emerging field of studying glycans (sugars) and glycoconjugates that are involved in almost all biological processes, from fine-tuning glycoprotein function to protein-protein interactions, cell signaling, immune response, host-pathogen interactions, etc. However, due to the chemical complexity of glycans and analytical challenges this exciting field was lagging behind other areas of biology. With technological advancements, a growing number of glycans' functions are being discovered and the study of glycans is becoming a cutting-edge discipline in basic and clinical research.

Despite recent developments in the glycobiology field, many aspects of the glycosylation process remain unknown, both in a healthy human organism and in pathological states. Interindividual variability in glycome composition is substantial and this strongly affects many physiological functions. Moreover, human glycome is dynamic and changes with physiological triggers, immune challenges, and disease. Altered glycosylation is consequently a subject of disease biomarker research and a target for therapeutic interventions. On the other hand, the properties of glycosylated biotherapeutics are significantly affected by their glycans.

The Role of Glycosylation in Health and Disease provides a comprehensive overview of the types and functions of glycans in a healthy human organism as well as their role in the pathophysiology of different diseases. Written by the experts in the field, this book aims to bring the glycobiology field closer to students, researchers in life sciences, and professionals in the biopharmaceutical industry.

The first part covers the synthesis and biological functions of the major types of glycoconjugates, extracellular vesicles glycosylation and functions, as well as genetic and epigenetic regulation and heritability of the glycosylation process. The second part of the book explores aberrant glycosylation patterns in various diseases (e.g. neurodegenerative, autoimmune, inflammatory diseases, etc.), suboptimal health status, and aging. Also, recent findings on glycosylation importance in COVID-19 infection are covered.

We would like to thank all the contributors, who are leading researchers in the field of glycobiology, for their participation in creating this book. We would also like to thank Dr. Gonzalo Cordova, Associate Editor of the Biomedicine Cell Biology, Molecular Biology, Biochemistry and Biophysics, for his guidance and continuous support.

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Abbreviations

αMII	α-mannosidase II
α–syn	*alpha*-synuclein
2-DG	2-deoxy-D-glucose
2FF	2-fluorofucose
3D	three-dimensional
5-aza-CdR	5-aza-2'-deoxycytidine
AAL	*Aleuria aurantia* lectin
Ab	antibody
ACAST	anti-calcitostatin antibody
ACE2	angiotensin-converting enzyme 2
ACPA	anticitrullinated protein/peptide antibodies
ACR	albumin-to-creatinine ratio
AD	Alzheimer's disease
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ADE	antibody-dependent enhancement
ADSCs	adipose tissue-derived mesenchymal stem cells
AFA	anti-fibronectin antibody
AFP	alpha-fetoprotein
AGFI	adjusted goodness-of-fit index
AHA	American Heart Association
AIDS	acquired immune deficiency syndrome
AITD	autoimmune thyroid diseases
AKA	anti-keratin antibodies
ALG7	Asn-linked glycosylation 7
ALIX	ALG-2 interacting protein X
ALS	amyotrophic lateral sclerosis
ANA	anti-nuclear antibody
ANHA2	annexin A2
ANP	atrial natriuretic peptide
anti-CarP	anti-carbamylated protein
anti-GPV	anti-glucose-6 phosphate isomerase
anti-hnRNP/RA33	anti-heterogeneous nuclear ribonucleoprotein
anti-MCV	anti-mutated citrullinated vimentin
AOS	Adams–Oliver syndrome
APC	antigen presenting cell

APF	antiperinuclear factor
APP	amyloid protein precursor
ARDs	age-related diseases
ARF6	ADP-ribosylation factor 6
ARMMS	ARRDC1-mediated microvesicles
ARRDC1	arrestin domain containing protein 1
ASCA	anti-*Saccharomyces cerevisiae* antibodies
AUC	area under the curve
B3GALT5	β1,3-galactosyltransferase isoenzyme 5
B3GNT6	core-3 synthase
B4GALNT2	β-1,4-*N*-acetyl-galactosaminyltransferase 2
BBB	blood brain barrier
BCG	Bacillus Calmette and Guérin
BDNF	brain-derived neurotrophic factor
BiTEs	bispecific T-cell engagers
BMI	body mass index
Bn	bacillus-negative
BP	blood pressure
C1GALT1	core-1 synthase
C1q	complement component 1q
CAD	coronary artery disease
CANX	calnexin
CAR-Ts	chimeric antigen receptor T cells
CARL	calreticulin
CAS	cerebral arterial stenosis
Cav-1	caveolin-1
CD	Crohn's disease
CD-MPR	cation-dependent Man-6-P receptors
CDC	complement-mediated cytotoxicity
CDG	congenital disorders of glycosylation
Cer	ceramide
CFA	confirmatory factor analysis
CFS	chronic fatigue syndrome
CGN	cerebellar granule neurons
CH2	constant heavy 2 domain
ChIP	chromatin immunoprecipitation
CI-MPR	cation-independent Man-6-P receptor
CKD	chronic kidney disease
CLPs	common lymphocyte progenitors
CLR	C-type lectin receptor
CMP	cytidine monophosphate
CNS	central nervous system
COACS	China suboptimal health cohort study
ConA	Concanavalin lectin
COPII	coat protein complex II
COPD	chronic obstructive pulmonary disease
CoV	coronavirus
COVID-19	coronavirus disease 2019

CP	convalescent plasma
CQ	chloroquine
CRP	C-reactive protein
CS	chondroitin sulfate
CS-A	chondroitin-4-sulfate
CS-B	chondroitin sulfate B, sulfate
CS-C	chondroitin-6-sulfate
CS-D	chondroitin-2,6-sulfate
CS-E	chondroitin-4,6-sulfate
CS-K	chondroitin-3-sulfate
CSF	colony stimulating factor
CSF	cerebrospinal fluid
CSPG	chondroitin sulfate proteoglycan
CT	cytoplasmic tail
CTD	C-terminal domain
CTL	C-type lectin
CTLA-4	cytotoxic T lymphocyte antigen-4
Ctx-B	cholera toxin-subunit B
CVD	cardiovascular disease
CVH	cardiovascular health
DAMP	damage-associated molecular pattern
DBP	diastolic blood pressure
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin 1
DDD	Dowling-Degos disease
df	degree of freedom
DIT	3,5-diiodothyrosine
DLB	dementia with Lewy bodies
DLL	Delta-like ligand
DN1	double negative 1
DNMTs	DNA methyltransferases
Dol-P	dolicol-phosphate
DP	degree of polymerization
DP cells	double-positive cells
DPM	Dol-P-Man
DPP4	dipeptidyl peptidase 4
DS	dermatan sulfate, Down syndrome
DSA-FACE	DNA sequencer-assisted, fluorophore-assisted carbohydrate electrophoresis
dsDNA	double-stranded DNA
EAE	experimental autoimmune encephalomyelitis
EBL	Elderberry lectin
EC	endothelial cells
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDEM	ER degradation-enhancing α-mannosidase-like 2

EGF	epidermal growth factor
eGFR	estimated glomerular filtration rate
EGFR	EGF receptor
EM	cryoelectron microscopy
ENGase	endo-β-*N*-acetylglucosaminidase
Env	envelope
EOGT	EGF-domain-specific *O*-GlcNAc transferase
EPIC	European Prospective Investigation of Cancer study
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ERK	extracellular signal-regulated kinase
ERK1/2	extracellular signal-regulated protein kinases 1 and 2
ERManI	ER-mannosidase I
ESCRT	endosomal sorting complexe required for transport
ETPs	early thymocyte progenitors
EV	extracellular vesicle
Fab	fragment, antigen binding
FAPP2	four-phosphate adaptor protein 2
FBG	fasting blood glucose
FBS	fetal bovine serum
Fc	fragment, crystallizable
FcR	Fc receptor
FcγRIIIA	Fcγ receptor IIIA
FGF	fibroblast growth factor
FINRISK	Finland Cardiovascular Risk Study
Fringe	β-1,3-*N*-acetylglucosaminyltransferase
Fut1	fucosyltransferase 1
FUT2	fucosyltransferase 2
FUT7	α-1,3-fucosyltransferase, FucT-VII
FUT8	fucosyltransferase 8
G0	agalactosylated glycans
G1	monogalactosylated glycans
G2	digalactosylated glycans
GAD65	glutamic acid decarboxylase
GAG	glycosaminoglycan
GalNAc-Ts	*N*-acetylgalactosamine transferases
GAS	group A Streptococcus
gal	galectin
GalCer	galactosylceramide
GC	germinal centre
GCase	β-glucocererebrosidase enzyme
GD	Gaucher's disease
GD	Grave's disease
GDM	gestational diabetes during pregnancy

GFI	goodness-of-fit index
GIP	glucose-dependent insulinotropic polypeptide
GlcCer	glucosylceramide
GlcNAcT-I	*N*-acetylglucosaminyltransferase I
GLP-1	glucagon-like peptide-1
GlycA	glycoprotein acetylation
GLUT2	glucose transporter-2
GLUT4	glucose transporter-4
GNE	UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase
GnT-I	*N*-acetylglucosaminyltransferase I
GnT-V	β-1,6-*N*-acetylglucosaminyltransferase V
GnT-IX	*N*-acetylglucosaminyltransferase IX
GO	gene ontology
GP	glycan peak
GPI	glycosylphosphatidylinositol
GRα	glucocorticoid receptor α
GSIS	glucose-stimulated insulin secretion
GSL	glycosphingolipids
GS	geriatric syndrome
GWAS	genome-wide association study
H. pylori	*Helicobacter pylori*
HA	hyaluronic acid
HA	hemagglutinin
hACE2	human angiontensin-converting enzyme 2
HAdV	human adenoviruses
HAND	HIV-associated neurocognitive disorders
HBP	hexosamine biosynthetic pathway
HBV	hepatitis B virus
HBVsAg	hepatitis B virus surface antigen
HC	healthy controls
HCC	hepatocellular carcinoma
hCG	human chorionic gonadotrophin
HCQ	hydroxychloroquine
HCV	hepatitis C virus
HD	Huntington's disease
HDAC	histone deacetylase
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HE	hemagglutinin-esterase
HexCer	hexosilceramide
HFD	high-fat diet
hIBM	hereditary inclusion-body myositis
HILIC-UHPLC-FLD	hydrophilic-interaction ultra-high-performance liquid chromatography with fluorescence detection
HILIC-UPLC	hydrophilic interaction ultra-performance liquid chromatography

hiPSC	human induced pluripotent stem cell
HIV-1	human immunodeficiency virus-1
HNF1A	hepatocyte nuclear factor 1-alpha
HNK-1	human natural killer-1
HP	heparin
HPLC	high-performance liquid chromatography
HPTLC	high-performance silica gel thin-layer chromatography
HS	heparan sulfate
HSBP	heparin sulfate binding proteins
hsCRP	high-sensitivity C-reactive protein
HSP90B1	heat shock protein 90 beta family member 1
HSPG	heparan sulfate proteoglycan
HT	Hashimoto's thyroiditis
HTCC	*N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride
IA-2	islet antigen-2
IAV	influenza A virus
IBD	inflammatory bowel disease
IC	immune complexes
iDC	immature dendritic cell
IFN	interferon
IgA1	immunoglobulin A1
IgG	immunoglobulin G
IIM	idiopathic inflammatory myopathies
IL	interleukin
ILC	innate lymphoid cell
ILC3	group 3 innate lymphoid cells
ILVs	intraluminal vesicles
IMPDH	inosine-5′-monophosphate dehydrogenase
IR	insulin receptor
IS	ischemic stroke
ITIM	immunoreceptor tyrosine-based inhibition motifs
iTreg	induced regulatory T cell
KGZ	Kirgiz
KO	knockout
KS	keratan sulfate
KZK	Kazakh
LabA	LacdiNAc- binding adhesin
LacCer	lactosylceramide
LAM	lipoarabinomannan
LCA	*Lens culinaris* agglutinin
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD	linkage disequilibrium
LDL	low-density lipoprotein

LDLR	low-density lipoprotein receptor
LDL-C	low-density lipoprotein cholesterol
lectin-ELISA	lectin-enzyme-linked immunosorbent assay
LEL	lectin from *Lycopersicon esculentum*
LFNG	Lunatic Fringe
LIMP-2	lysosomal integral membrane protein 2
LM	lipoarabinomannan
LMWH	low molecular weight heparin
lncRNA	long non-coding RNA
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
LRPs	low-density lipoprotein receptor-related proteins
LSDs	lysosomal storage disorders
M6PR	mannose-6-phosphate receptor
mAb	monoclonal antibody
MAG	myelin-associated glycoprotein
MALDI-QIT-TOF-MS/MS	matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MAL-II	*Maackia amurensi*s II lectin
ManLAM	mannose-capped lipoarabinomannan
ManR	mannose receptor
MAPK	mitogen-activated protein kinase
MASP	mannose-binding lectin-associated serine protease
MBL	mannose-binding lectin
MBP	mannose-binding protein
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
mDC	mature dendritic cell
ME	myalgic encephalomyelitis
MERS-CoV	Middle East respiratory syndrome coronavirus
MetS	metabolic syndrome
MFNG	Manic Fringe
MGAT1	*N*-acetylglucosaminyltransferase I
Mgat5	alpha-1,6-mannosylglycoprotein 6-beta-*N*-acetylglucosaminyltransferase A
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
miRNA	micro RNA
MIT	3-monoiodothyrosine
MLCK	myosin light chain kinase
moDCs	monocyte-derived dendritic cells

MODY	maturity onset diabetes of the young
MOG	myelin-associated basic oligodendrocytic protein
Mpro	main protease
MR	mannose receptor
MRH	mannose-6-phosphate receptor homology
mRNA	messenger RNA
MS	mass spectrometry
MS	multiple sclerosis
MSA	multiple system atrophy
MSCs	mesenchymal stem cells
M.tb	*Mycobacterium tuberculosis*
mTORC1	mammalian/mechanistic target of rapamycin complex 1
MZ	marginal zone
N-DNJ	*n*-butyldeoxynojirimycin
NA	neuraminidase
nAbs	neutralizing antibodies
NCS	neural stem cells
NDD	*n*eurodegenerative disease
NDV	Newcastle disease virus
NEC	neuroepithelial cells
NFTs	neurofibrillary tangles
NGF	nerve growth factor
NHC	β-D-*N*4-hydroxycytidine
NK	natural killer
NMR	nuclear magnetic resonance
NP-HPLC	normal phase high-pressure liquid chromatography
nSMase 2	neutral sphingomyelinase 2
NTD	N-terminal domain
OAA	agglutinin from *Oscillatoria Agardhii*
OGT	*O*-GlcNAc transferase
OGTT	oral glucose tolerance test
OligoGM1	GM1-oligosaccharide, $II^3Neu5Ac\text{-}Gg_4$
OST	oligosaccharyltransferase
PAD	peripheral artery disease
PAMP	pathogen-associated molecular pattern
PAMR1	peptidase domain-containing protein associated with muscle regeneration 1
pANCA	perinuclear anti-neutrophil cytoplasmatic antibodies
PBMCs	peripheral blood mononuclear cells
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PD-L1	programmed cell death 1 ligand 1
PG	proteoglycan
PHA-L	*Phaseolus vulgaris* lectin

PHA-L4	isolectin L4 from *Phaseolus vulgaris*
PIM	phosphatidyl-myo-inositol mannoside
PLD	phospholipase D
PLP	proteolipid protein
PLS	primary lateral sclerosis
PM	plasma membrane
PMN	polymorphonuclear cell
PNN	perineuronal net
POFUT1	protein *O*-fucosyltransferase 1
pre-B	precursor B cells
pro-B	progenitor B cells
pro-BNP	pro-brain natriuretic peptide
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PSP	progressive supranuclear palsy
PTC	papillary thyroid carcinoma
pZAP70	ZAP70 phosphorylation
P-ERK1/2	phosphorylated ERK1/2
P-TrkA	phosphorylated TrkA
RA	rheumatoid arthritis
rACE2	recombinant angiotensin-converting enzyme 2
RAG	recombination-activating genes
RANKL	receptor activator of nuclear factor kappa-B ligand
rBC2LCN	recombinant form of the lectin derived from *Burkholderia cenocepacia*
RBC	red blood cells
RBD	receptor-binding domain
RBM	receptor-binding motif
RdRp	RNA-dependent RNA polymerase
RF	rheumatoid factor
RFNG	Radical Fringe
RMSEA	root mean square error of approximation
RNPs	ribonucleoproteins
ROC	receiver operating characteristic
ROS	reactive oxygen species
S. pyogenes	*Streptococcus pyogenes*
S1P1	sphingosine-1-phosphate receptor 1
SAP	sphingolipid activator protein
SARS-CoV	severe acute respiratory syndrome coronavirus
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SBP	systolic blood pressure
SC	stratum corneum
SCA	spinocerebellar ataxia
SCORE	Systematic Coronary Risk Evaluation
sEV	small extracellular vesicles

SH3	src homology 3
SHS	Suboptimal Health Status
SHSQ-25	Suboptimal Health Status Questionnaire-25
sIBM	sporadic inclusion-body myositis
sIgA	secretory immunoglobulin A
Siglec	sialic acid-binding immunoglobulin (Ig)-like lectin
SLC9A9	solute carrier family 9 member A9
SLE	systemic lupus erythematosus
sLex	sialyl Lewis X
SM	sphingomyelin
Sm	Smith antigen
SMA	spinal muscular atrophy
SNEC	secondarily necrotic cell-derived material
SNP	single nucleotide polymorphism
SP cells	single positive cells
SP-D	surfactant protein D
Sph	sphingoids
SRBI	scavenger receptor-BI
S. *typhimurium*	*Salmonella typhimurium*
ST3Gal4	α2,3-sialyltransferase
ST3Gal6	α2,3-sialyltransferase
ST6GAL1	β-galactoside α-2,6-sialyltransferase 1
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
T3	L-triiodothyronine
T4	L-thyroxine (tetraioothyronine)
TAILS	terminal amine isotopic labelling of substrates
TB	*tuberculosis*
TCR	T cell receptor
TCRβ	β chain of the TCR
TECs	thymic epithelial cells
TG	triglyceride
Tg	thyroglobulin
TgAb	anti-Tg IgG
TGF	transforming growth factor
TGN	trans Golgi network
Th17	T helper-17
TJK	Tajik
TLR2	Toll-like receptor 2
TNF	tumor necrosis factor
TPO	thyroid peroxidase (thyroperoxidase)
Trk	neurotrophin tyrosine kinase receptor
TSA	Trichostatine A
TSG101	tumor susceptibility gene 101
TSH	thyroid stimulating hormone (thyrotropin)
TSHR	thyrotropin receptor
TSPs	thymus seeding progenitors

TSRs	thrombospondin type-I repeats
TSS	transcription start site
UC	ulcerative colitis
UCE	*N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase; uncovering enzyme
UDP	uridine diphosphate
UEA-I	*Ulex europaeus* agglutinin
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UHPLC	ultra-high-performance liquid chromatography
UIG	Uygur
UMS	unexplained medical syndrome
uPA	urokinase-type plasminogen activator
UPEC	uropathogenic *E. coli*
UPLC-ESI-QqQ-MS	ultra-performance liquid chromatography electrospray ionization triple quadrupole mass spectrometry
UTI	urinary tract infection
VEGFR	vascular endothelial growth factor receptor
VH	variable heavy
VIP36	vesicular integral membrane 36 kDa protein
WGA	wheat germ agglutinin
WHO	The World Health Organization
WHR	waist-to-hip ratio
Wif1	Wnt inhibitory factor 1
ZnT8	zinc transporter 8

Sugars

Fuc	fucose
Gal	galactose
GlcA	glucuronic acid
GlcN	glucosamine
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
IdoA	iduronic acid
LacNAc	*N*-acetyllactosamine, Galβ1,4GlcNAc disaccharide unit
Man	mannose
ManNAc	*N*-acetylmannosamine
Neu5Ac, Sia	*N*-acetylneuraminic acid, sialic acid

Part I

Importance and Functions of the Human Glycome

N-Glycosylation

1

Tetsuya Hirata and Yasuhiko Kizuka

Abstract

N-glycosylation is a highly conserved glycan modification, and more than 7000 proteins are *N*-glycosylated in humans. *N*-glycosylation has many biological functions such as protein folding, trafficking, and signal transduction. Thus, glycan modification to proteins is profoundly involved in numerous physiological and pathological processes. The *N*-glycan precursor is biosynthesized in the endoplasmic reticulum (ER) from dolichol phosphate by sequential enzymatic reactions to generate the dolichol-linked oligosaccharide composed of 14 sugar residues, $Glc_3Man_9GlcNAc_2$. The oligosaccharide is then en bloc transferred to the consensus sequence N-X-S/T (X represents any amino acid except proline) of nascent proteins. Subsequently, the *N*-glycosylated nascent proteins enter the folding step, in which *N*-glycans contribute largely to attaining the correct protein fold by recruiting the lectin-like chaperones, calnexin, and calreticulin. Despite the *N*-glycan-dependent folding process, some glycoproteins do not fold correctly, and these misfolded glycoproteins are destined to degradation by proteasomes in the cytosol. Properly folded proteins are transported to the Golgi, and *N*-glycans undergo maturation by the sequential reactions of glycosidases and glycosyltransferases, generating complex-type *N*-glycans. *N*-Acetylglucosaminyltransferases (GnT-III, GnT-IV, and GnT-V) produce branched *N*-glycan structures, affording a higher complexity to *N*-glycans. In this chapter, we provide an overview of the biosynthetic pathway of *N*-glycans in the ER and Golgi.

Keywords

N-glycan · Oligosaccharyltransferase · Glycosyltransferase

1.1 Introduction

N-glycosylation is a major posttranslational modification that occurs in the secretory pathway and is highly conserved among eukaryotes (Varki 2017; Moremen et al. 2012). It has been reported that more than 7000 human proteins are *N*-glycosylated (Sun et al. 2019). These *N*-glycans are critically involved in processes such as protein folding, protein trafficking, and signal transduction (Stanley et al. 2015; Zhao et al. 2008).

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Therefore, *N*-glycosylation serves various physiological phenomena such as development, neurogenesis, and immunity by regulating the function of proteins. Furthermore, alterations to *N*-glycan structures are involved in disease development and aggravation (Ohtsubo and Marth 2006; Kizuka and Taniguchi 2016; Pinho and Reis 2015; Lauc et al. 2016). Thus, understanding the biosynthetic pathway of *N*-glycans and elucidating the mechanisms of how *N*-glycan structures are regulated in cells and are dysregulated in disease states are important.

N-Glycans are biosynthesized in the endoplasmic reticulum (ER) and the Golgi apparatus by stepwise reactions involving glycosidases and glycosyltransferases (Stanley et al. 2015). The process of *N*-glycosylation is divided into four parts: (1) assembly of an *N*-glycan precursor composed of 14 sugar residues, $Glc_3Man_9GlcNAc_2$, in the ER; (2) en bloc transfer of $Glc_3Man_9GlcNAc_2$ to nascent proteins; (3) *N*-glycosylation-dependent protein folding; and (4) maturation of *N*-glycans in the Golgi to generate complex-type *N*-glycans. All these steps are tightly controlled by many factors, and diverse *N*-glycan structures are synthesized in cell type-, protein-, and even glycosylation site-specific manners, which allows for dynamic and specific regulation of glycoprotein functions.

In this chapter, we summarize the basics of *N*-glycan biosynthesis in the ER and Golgi. We also describe the biological functions of *N*-glycans with a particular focus on branched structures. Please also see other chapters where *N*-glycan-related diseases are described in detail.

1.2 *N*-Glycosylation Process in the ER

1.2.1 Biosynthesis of the *N*-Glycan Precursor in the ER

Biosynthesis of *N*-glycan in eukaryotes starts in the ER from dolichol phosphate (Dol-P), which is a highly hydrophobic polyisoprenoid molecule (Fig. 1.1). The first step is catalyzed on the cytosolic side of the ER by Asn-linked glycosylation 7 (ALG7, also termed DPAGT1 in mammalian cells), a GlcNAc-1-phosphotransferase that transfers GlcNAc-1-P from UDP-GlcNAc to Dol-P, generating Dol-P-P-GlcNAc (Eckert et al. 1998; Rine et al. 1983). Tunicamycin, the most popular inhibitor of *N*-glycan biosynthesis, inhibits this enzyme, resulting in the complete loss of *N*-glycosylation in cells (Schwarz et al. 1979; Kaushal and Elbein 1994). Tunicamycin is highly toxic to cells because of severe ER stress caused by non-glycosylation of a huge number of proteins, and prolonged treatment with this inhibitor leads to cell death. Recent structural analyses of DPAGT1 revealed the inhibition mechanisms of tunicamycin, which provided the molecular basis for the development of novel antimicrobial drugs (Yoo et al. 2018; Dong et al. 2018). The second step is mediated by the ALG13/14 heterocomplex, which transfers GlcNAc, generating Dol-P-P-$GlcNAc_2$. ALG13 is a catalytic subunit lacking a transmembrane domain, whereas ALG14 is a non-catalytic transmembrane protein that recruits ALG13 to the ER membrane (Bickel et al. 2005; Gao et al. 2005; Gao et al. 2008). Furthermore, DPAGT1 and ALG13/ALG14 form a multienzyme complex (Noffz et al. 2009). Next, ALG1 transfers Man from GDP-Man to generate Dol-P-P-$GlcNAc_2$-Man (Couto et al. 1984). ALG2 catalyzes the next two Man-transfer steps to yield Dol-P-P-$GlcNAc_2$-Man_3, and ALG11 transfers two Man residues at the next two steps to generate Dol-P-P-$GlcNAc_2$-Man_5 (Jackson et al. 1993; Cipollo et al. 2001; O'Reilly et al. 2006). These three enzymes, ALG1, ALG2, and ALG11, form a multienzyme complex (Gao et al. 2004). Because the remaining reactions are carried out on the luminal side of the ER, Dol-P-P-$GlcNAc_2$-Man_5 must be flipped into the ER lumen. RFT1 was initially reported to be the responsible flippase in yeast (Helenius et al. 2002), but subsequent studies reported contradictory results (Sanyal et al. 2008; Rush et al. 2009). Therefore, the flipping mechanism and how RFT1 is involved in this process are unresolved.

Dol-P-P-$GlcNAc_2$-Man_5 in the ER lumen is sequentially modified by three mannosyltransferases, ALG3, ALG9, and ALG12, which commonly use Dol-P-Man as a donor substrate to

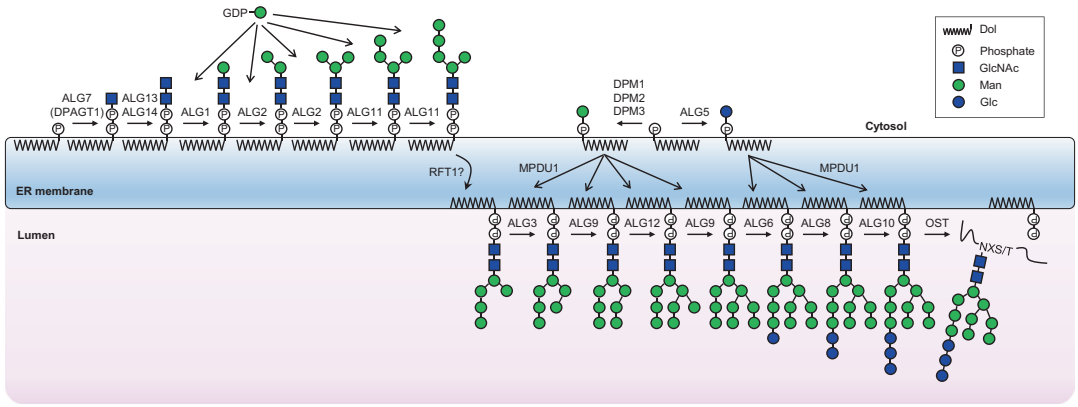

Fig. 1.1 Biosynthesis pathway of *N*-glycan in the ER Biosynthesis of mammalian *N*-glycan starts from dolichol phosphate (Dol-P) on the cytosolic face of the ER. After seven sequential reactions, the *N*-glycan precursor is flipped to the luminal side of the ER by an unknown mechanism. Subsequently, an additional seven reactions complete *N*-glycan assembly to generate $Glc_3Man_9GlcNAc_2$. OST en bloc transfers these 14 sugar residues to nascent glycoproteins at the consensus sequence, NXS/T. Two dolichol-linked donor substrates, Dol-P-Man and Dol-P-Glc, are synthesized on the cytosolic side of the ER. These molecules are also flipped to the ER lumen by an unidentified flippase. Glycan symbols follow the Symbol Nomenclature for Glycans (Varki et al. 2015)

generate Dol-P-P-$GlcNAc_2$-Man_9 (Frank and Aebi 2005; Aebi et al. 1996; Burda et al. 1999; Sharma et al. 2001). Dol-P-P-$GlcNAc_2$-Man_9 is then modified with Glc by ALG6, ALG8, and ALG10, which transfer Glc from Dol-P-Glc to give Dol-P-P-$GlcNAc_2$-Man_9-Glc_3, the complete *N*-glycan precursor (Reiss et al. 1996; Stagljar et al. 1994; Burda and Aebi 1998). All enzymes that use Dol-P-Man or Dol-P-Glc are classified as GT-C fold multi-transmembrane glycosyltransferases (Albuquerque-Wendt et al. 2019). Because of the difficulty of crystallizing these transmembrane proteins, the three-dimensional (3D) crystal structures of GT-C fold enzymes have been barely studied. Recently, cryoelectron microscopy (EM) was used to solve the 3D structure of ALG6, which revealed how a GT-C fold enzyme recognizes acceptor and donor substrates (Bloch et al. 2020).

Dol-P-Man is synthesized from GDP-Man by a Dol-P-Man (DPM) complex in mammalian cells, which is composed of DPM1, DPM2, and DPM3, and DPM1 is the catalytic subunit (Maeda and Kinoshita 2008) (Fig. 1.1). As DPM2 and DPM3 but not DPM1 are transmembrane proteins, DPM2 and DPM3 are essential for recruiting and tethering DPM1 to the ER membrane (Maeda et al. 2000; Maeda et al. 1998). Dol-P-Glc is synthesized by ALG5 (Heesen et al. 1994). As Dol-P-Man and Dol-P-Glc are synthesized in the cytosol, both donors are likely flipped to the ER lumen. Although MPDU1 is involved in the utilization of these donors in the ER lumen (Camp et al. 1993), the flippase involved in this process is still under discussion with controversial results.

1.2.2 En Bloc Transfer of the *N*-Glycan

After synthesis of the complete *N*-glycan precursor, Dol-P-P-$GlcNAc_2$-Man_9-Glc_3, the oligosaccharide ($Glc_3Man_9GlcNAc_2$) is en bloc transferred to the Asn residue in the consensus sequence N-X-S/T (X represents any amino acid except proline) of nascent proteins by oligosaccharyltransferase (OST) (Fig. 1.1). OST is a multisubunit protein complex in the ER. Mammalian cells express two types of OSTs, OSTA and

OSTB, in which STT3A and STT3B are the catalytic subunits, respectively (Cherepanova et al. 2016). OSTA transfers *N*-glycans by a co-translational mechanism, whereas OSTB transfers *N*-glycans by both co-translational and posttranslational mechanisms (Ruiz-Canada et al. 2009). The other subunits are common to both OST complexes or specific to each OST. Ribophorin I, ribophorin II, OST4, OST48, TMEM258, and DAD1 are the common subunits, whereas DC2 and KCP2 are specific to OSTA, and MAGT1 and TUSC3 are found only in OSTB (Kelleher et al. 2003; Roboti and High 2012; Blomen et al. 2015). The two OST complexes have slightly distinct specificities toward dolichol-linked oligosaccharides. In vitro experiments showed that OSTA transfers only fully assembled *N*-glycan, whereas OSTB transfers fully assembled *N*-glycan and an incomplete *N*-glycan lacking three Glc residues at lower efficiency (Kelleher et al. 2003).

The recent cryo-EM structure of mammalian OSTA complexed with the Sec61 translocon and a ribosome shows that OSTA is connected to the ribosome via the C-terminus of ribophorin I and to Sec61 via the DC2 subunit (Braunger et al. 2018). This observation was confirmed by another cryo-EM study of human OSTA and OSTB (Ramirez et al. 2019). Although human OSTA and OSTB show high structural similarity, there are two marked differences. First, in contrast to the similar arrangements of the transmembrane helices of STT3A and STT3B, there is a difference in the protein surfaces for the specific interaction with DC2 or MAGT1, resulting in steric hindrance with other partners (Ramirez et al. 2019). This enables the specific interaction of OSTA but not OSTB with a translocon. The second difference is the C-terminal structure of ribophorin I. The C-terminus of ribophorin I in human OSTA forms four helices on the cytosolic side, whereas no such feature was observed in human OSTB, indicating high flexibility of this region in OSTB (Ramirez et al. 2019). This ribophorin I feature allows OSTA to interact with ribosomes. The specific interactions with a translocon and a ribosome enable OSTA to transfer *N*-glycan to nascent proteins in a co-translational manner. Some consensus sequences can be skipped by OSTA for the following four reasons: (1) the sequence is located in close proximity to the N- or C-terminus; (2) two consensus sequences are too close; (3) the sequence is close to a cysteine residue that forms a disulfide bond; and (4) unknown reasons (Cherepanova et al. 2016). OSTB transfers *N*-glycans at these skipped sites in a posttranslational manner. MAGT1 and TUSC3, the two subunits specific to OSTB, both contain a thioredoxin motif, CXXC. The active site of this motif in MAGT1 is required for *N*-glycan transfer at the sequence with a disulfide bond and also for other skipped sequences, indicating that MAGT1 functions to recruit glycoprotein substrates (Cherepanova et al. 2014). TUSC3 may have functional redundancy to MAGT1 because TUSC3 is incorporated into OST3B in MAGT1-defective cells (Cherepanova and Gilmore 2016).

Recently, SELT was identified as an OSTA-specific subunit in pituitary cells (Hamieh et al. 2017). SELT is a thioredoxin-like enzyme with a selenocysteine in its thioredoxin motif. Depletion of SELT leads to STT3A instability and hypoglycosylation of specific proteins harboring disulfide bonds. SELT may enhance OSTA activity for cysteine-rich proteins through its thioredoxin-like motif, like MAGT1 or TUSC3 in OST3B (Hamieh et al. 2017).

1.2.3 Quality Control of Glycoproteins by *N*-Glycan

After the transfer of *N*-glycans, glycoproteins enter the quality control process. This process is divided into two phases: the folding step and degradation step of misfolded proteins (Fig. 1.2). *N*-glycans contribute largely to these processes.

The first step is the removal of the Glc residue from the transferred *N*-glycans, Dol-P-P-$GlcNAc_2$-Man_9-Glc_3, by glucosidase I (MOGS) (Bause et al. 1989). The reaction occurs immediately after the transfer of fully assembled *N*-glycans to generate Dol-P-P-$GlcNAc_2$-Man_9-Glc_2. The next enzyme, glucosidase II, trims the residual two Glc residues by different kinetics to gen-

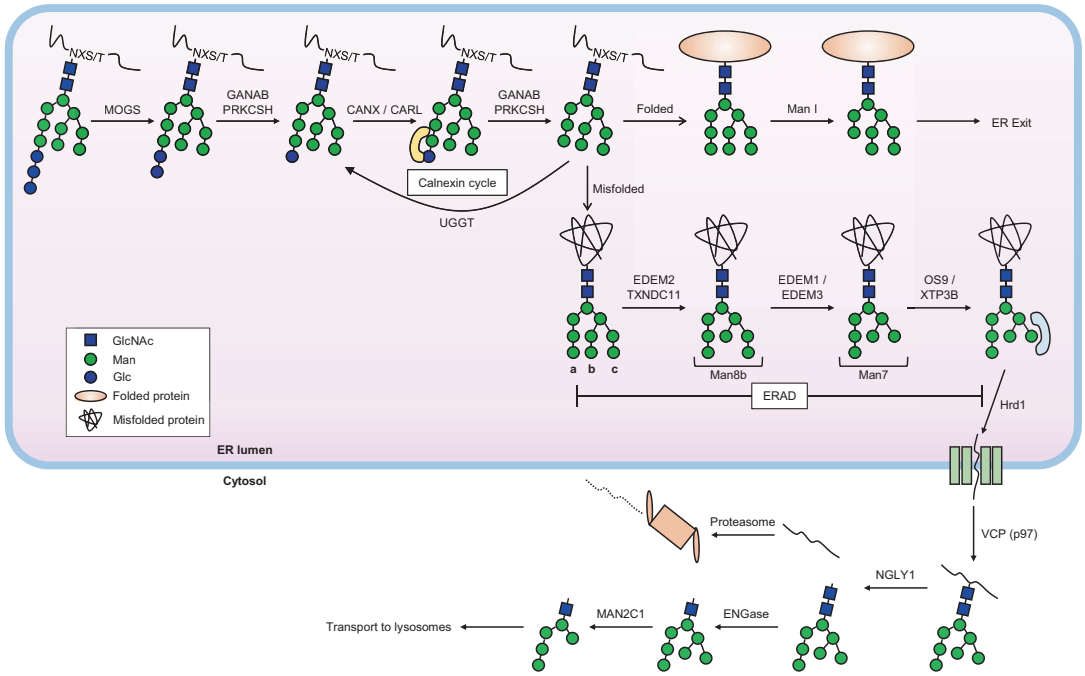

Fig. 1.2 *N*-Glycan-dependent quality control of glycoproteins
Nascent glycoproteins enter a folding step soon after *N*-glycosylation. After sequential Glc trimming, two lectin-like chaperones, CANX and CARL, facilitate *N*-glycan-dependent folding. UGGT transfers a Glc residue to un-glucosylated oligomannose-type *N*-glycans. This process is called the calnexin cycle. If glycoproteins are properly folded, they are allowed to exit from the ER. If glycoproteins are finally misfolded, despite several rounds of the calnexin cycle, they enter the ERAD process. Misfolded glycoproteins are retrotranslocated into the cytosol after sequential Man trimming. NGLY1 removes *N*-glycans from misfolded glycoproteins, generating free *N*-glycans and misfolded proteins. Misfolded proteins are subsequently degraded by proteasomes, while the free *N*-glycans are degraded by sequential reactions in the cytosol and finally degraded completely in lysosomes

erate Dol-P-P-GlcNAc$_2$-Man$_9$ (Alonso et al. 1991). The second reaction by glucosidase II occurs much slower than the first reaction and acts as a folding timer. Because two major ER chaperones, calnexin (CANX) and calreticulin (CARL), recognize Dol-P-P-GlcNAc$_2$-Man$_9$-Glc but not its de-glucosylated form, the slower production of Dol-P-P-GlcNAc$_2$-Man$_9$ defines the folding time (Caramelo and Parodi 2015). Glucosidase II acts as a heterodimer composed of α and β subunits (Treml et al. 2000). The α subunit (GANAB) has catalytic activity, whereas the β subunit (PRKCSH) contains the mannose-6-phosphate receptor homology (MRH) domain, which is important for Glc trimming by the α subunit (Hu et al. 2009). If folding of the glycoprotein is incomplete, *N*-glycans are re-glucosylated by UDP-glucose:glycoprotein glucosyltransferase (UGGT), and then glycoproteins are further folded by CANX and CARL (Roth and Zuber 2017). This glucosylation-mediated folding process is called the calnexin cycle. UGGT is a large glycosyltransferase with a C-terminal glycosyltransferase domain and an N-terminal folding sensor domain (Tessier et al. 2000). The crystal structure of the N-terminal domain of UGGT demonstrated that it contains four adjacent thioredoxin-like domains, and the overall structure adopts a C-shape structure with a flexible modular architecture. The central cavity exposes a highly hydrophobic surface that may be involved in the recognition of the unfolded glycoprotein substrates (Satoh et al. 2017). By combining glucosidase II and UGGT activities,

the calnexin cycle undergoes several rounds to facilitate the proper folding of glycoproteins. If folding finally fails, misfolded glycoproteins are degraded by ER-associated degradation (ERAD) (Roth and Zuber 2017). If folding is achieved, properly folded glycoproteins are allowed to exit from the ER.

The first step of the ERAD process is the trimming of Man on the b-arm by α-mannosidase (Fig. 1.2). Although mammalian ER-mannosidase I (ERManI) was considered to play a central role in this trimming step as in the case of its yeast homolog (Hosokawa et al. 2003; Slominska-Wojewodzka and Sandvig 2015), recent studies showed that another mannosidase is likely to be responsible for this step in mammals. ER degradation-enhancing α-mannosidase-like 2 (EDEM2) has been shown recently to be a key player that initiates the Man trimming of misfolded proteins to generate the Man8b structure (Ninagawa et al. 2014). EDEM2 was initially considered to be a lectin because of the difficulty in measuring enzyme activity in vitro (Mast et al. 2005). However, a recent study elegantly demonstrated its in vitro enzyme activity (George et al. 2020). EDEM2 is covalently bound to TXNDC11, a thioredoxin domain-containing protein, and purified EDEM2 with TXNDC11 clearly shows Man trimming activity (George et al. 2020). Consistent with this, EDEM2- or TXNDC11-depleted cells accumulate Man9 structures, causing defects in the efficient degradation of misfolded glycoproteins in cells (George et al. 2020). A Man residue on the c-arm of Man8b glycans is further trimmed by EDEM1 and EDEM3 to generate the Man7 structure (Hirao et al. 2006; Olivari et al. 2006; Hosokawa et al. 2010; Ninagawa et al. 2014). The Man7 structure is recognized by ER-resident lectins OS-9 and XTP-3B, which guide misfolded glycoproteins to the retrotranslocon (Roth and Zuber 2017). HRD1 is a major retrotranslocon in the ER and also functions as an E3 ubiquitin ligase to ubiquitinate the unfolded glycoproteins on the cytosolic face (Carvalho et al. 2010; Baldridge and Rapoport 2016; van den Boom and Meyer 2018). Hrd1 forms a multiprotein complex that includes OS-9 and DERL1. The 3D structures of yeast Hrd1 complexes were recently resolved by cryo-EM and clearly show that Hrd1 does form a channel for retrotranslocation (Wu et al. 2020; Schoebel et al. 2017). This work provides detailed mechanistic insights into how Hrd1 recognizes misfolded glycoproteins and facilitates retrotranslocation. The VCP/p97 complex, an AAA + -ATPase family comprised of a homohexamer, binds to the ubiquitinated substrates and pulls them out onto the cytosolic face (Stolz et al. 2011; Schuberth and Buchberger 2005; Neuber et al. 2005). In the cytosol, misfolded glycoproteins are de-*N*-glycosylated by NGLY1 and finally degraded by proteasomes (Suzuki 2016). The released *N*-glycans are further processed in the cytosol and eventually degraded in lysosomes, as described below.

N-Glycans on properly folded proteins are also trimmed by α-mannosidases and transported to the ER-Golgi intermediate compartment (ERGIC) and the Golgi. The coat proteins of COP-II vesicles can directly capture transmembrane proteins harboring cytosolic regions, whereas soluble secretory proteins have no direct interaction with the COP-II coat, thereby requiring a cargo receptor for efficient packaging into COP-II vesicles. ERGIC-53, a leguminous (L)-type lectin, is a well-characterized cargo receptor for secretory glycoproteins (Zhang et al. 2009). ERGIC-53 is a transmembrane protein with a lectin domain on the luminal side, and both ER-exit and ER-retrieval motifs (KKFF motif) are located within the cytosolic region. ERGIC-53 binds to oligomannose-type *N*-glycans on its cargo proteins and facilitates the formation of COP-II vesicles by recruiting COP-II coat proteins via an FF motif, which leads to the packaging of the clients into COP-II vesicles (Appenzeller et al. 1999). A KK motif serves as an ER-retrieval signal as it recruits COP-I coat proteins. These features allow ERGIC-53 to shuttle between the ER and the ERGIC or the Golgi. The binding of ERGIC-53 to *N*-glycans is Ca^{2+}- and pH-dependent (Itin et al. 1996; Appenzeller-Herzog et al. 2004). Neutral pH is suitable for ERGIC-53 to bind to *N*-glycans. This feature is important for efficient cargo binding in the ER and release in the ERGIC or the Golgi, because

the pH in the ER and Golgi is neutral and acidic, respectively. The other L-type lectin, vesicular integral membrane 36 kDa protein (VIP36), also serves as a glycoprotein cargo receptor. VIP36 also binds to *N*-glycans in a Ca^{2+}- and pH-dependent manner. However, in contrast to ERGIC-53, the affinity for *N*-glycans is highest under acidic conditions (pH 6.5), indicating that VIP36 mainly functions in the Golgi (Kamiya et al. 2005). Therefore, VIP36 likely has a role in the retrograde transport of misfolded proteins from the Golgi to the ER, which may contribute to another quality control system (Reiterer et al. 2010).

1.2.4 Degradation of Free *N*-Glycan Produced in the Cytosol

As described above, *N*-glycans are removed from misfolded glycoproteins and degraded by sequential reactions in the cytosol (Fig. 1.2). The first reaction, the removal of *N*-glycans from misfolded proteins, is carried out by NGLY1 to produce free *N*-glycans (Suzuki et al. 2000). The produced *N*-glycans are further trimmed by cytosolic endo-β-*N*-acetylglucosaminidase (ENGase) to remove the GlcNAc residue at the reducing end (Suzuki et al. 2002). Then, cytosolic α-mannosidase (MAN2C1) sequentially removes four Man residues on the α1,6-Man branch (both b and c arms), generating free $GlcNAc_1Man_5$ (Costanzi et al. 2006; Suzuki et al. 2006). As these reactions all occur in the cytosol, this degradation process is called non-lysosomal degradation. After this process, it is hypothesized that $GlcNAc_1Man_5$ is translocated into lysosomes and completely degraded. Although this translocation is known to be an ATP-dependent process (Saint-Pol et al. 1997; Saint-Pol et al. 1999), the responsible translocase has not been identified.

Degradation of cytosolic free *N*-glycans is significantly important to our health because patients harboring mutations in the *NGLY1* allele show severe symptoms (Need et al. 2012; Enns et al. 2014; Lam et al. 2017). In addition, *Ngly1*-knockout mice show severe cardiac defects before birth, leading to embryonic lethality (Fujihira et al. 2017). Additional knockout of *ENGase* in *Ngly1*-deficient mice, however, partially restores their lethality (Fujihira et al. 2017). In line with this notion, ENGase catalyzes the de-*N*-glycosylation reaction in NGLY1-deficient cells, producing *N*-GlcNAc proteins that easily aggregate, whereas NGLY1-ENGase-double deficient cells no longer present these phenomena (Huang et al. 2015a), suggesting that ENGase inhibition has the potential to mitigate symptoms of NGLY1 deficiency. Moreover, de-*N*-glycosylation by NGLY1 was recently found to be required for the activation of the ER-resident transcriptional factor, NRF1 (Lehrbach et al. 2019; Tomlin et al. 2017). Because NRF1 plays critical roles in the induction of proteasomal genes, this NGLY1-NRF1 axis is posited to be one of the physiological functions of NGLY1 and a novel therapeutic target for NGLY1 deficiency.

1.3 Biosynthesis Pathway of *N*-Glycan in the Golgi

1.3.1 Maturation Process of *N*-Glycan in the Golgi

N-Glycans are further processed in the Golgi and converted to the mature form (Fig. 1.3). The initial reaction in the Golgi is Man trimming. There are four mannosidases in the Golgi. Three of them are called Golgi α1,2-mannosidases (Golgi ManI, encoded by *MAN1A1*, *MAN1A2*, and *MAN1C1*), and the other one is ER ManI (encoded by *MAN1B1*) (Bause et al. 1993; Herscovics et al. 1994; Tremblay et al. 1998; Tremblay and Herscovics 2000; Gonzalez et al. 1999). Although ER ManI was found in the ER in an early study (Gonzalez et al. 1999), a recent study also reported its localization in the Golgi (Pan et al. 2011). Golgi ManI is postulated to be a main player for generating $GlcNAc_2$-Man_5, and a recent study indicates the contribution of ER ManI to this process (Jin et al. 2018). Two major mannosidase inhibitors, kifunensine and deoxymannojirimycin, inhibit these enzymes, resulting in the accumulation of oligomannose-type glycans. After the formation of $GlcNAc_2$-Man_5, *N*-acetylglucosaminyltransferase I (GnT-I, MGAT1) transfers a GlcNAc residue from UDP-GlcNAc

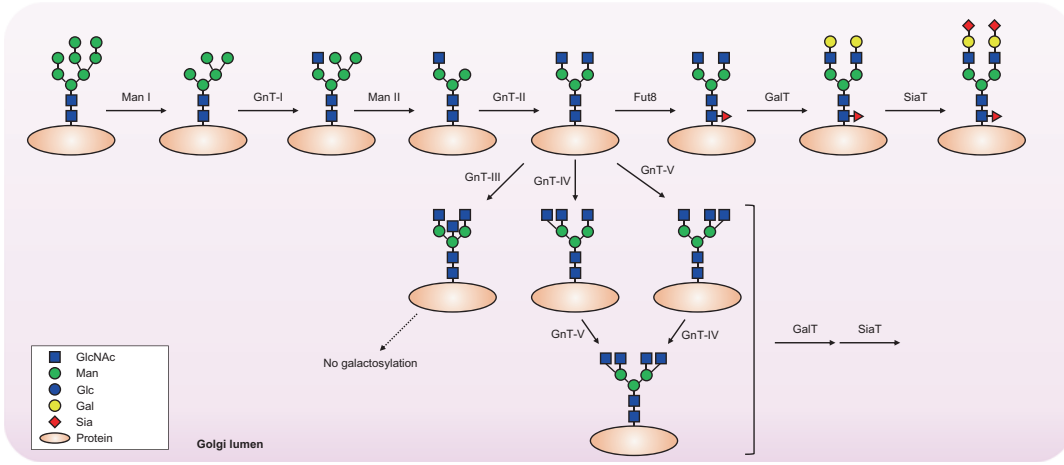

Fig. 1.3 Maturation process of *N*-glycan in the Golgi
The maturation process of *N*-glycan in the Golgi starts with the trimming of Man residues. Subsequently, GlcNAc is transferred by GnT-I, followed by further Man trimming by ManII. GnT-II transfers a GlcNAc residue at the newly exposed Man residue. GlcNAc branches are produced by three GnTs, GnT-III, GnT-IV, and GnT-V, which produce bisecting GlcNAc, the β1,4-GlcNAc branch and the β1,6-GlcNAc branch, respectively. *N*-glycans are further modified by other types of sugars, including Fuc (core Fuc), β1,3- or β1,4-Gal, and α2,3- or α2,6-Sia. The presence of bisecting GlcNAc hinders the production of these modifications

onto the α1,3 Man via the β1,2-linkage to generate $GlcNAc_2$-Man_5-GlcNAc (Kumar et al. 1990). This GnT-I function is a prerequisite for the subsequent action of enzymes. Golgi α-mannosidase II (Golgi ManII) sequentially removes two Man residues, generating $GlcNAc_2$-Man_3-GlcNAc (Tulsiani et al. 1982b). The mannosidase reaction occurs immediately after the GlcNAc transfer because GnT-I and Golgi ManII form a complex. Swainsonine inhibits Golgi ManII, leading to the loss of complex-type glycans following treatment with this inhibitor (Tulsiani et al. 1982a). GnT-II (MGAT2) transfers GlcNAc on newly exposed Man, generating $GlcNAc_2$-Man_3-$GlcNAc_2$. The structure is called bi-antennary and is a common core structure of complex-type glycans. Other GnTs, GnT-III (MGAT3), GnT-IV (MGAT4A and MGAT4B), and GnT-V (MGAT5), produce further GlcNAc branches in mammals. GnT-IV produces a β1,4-linked GlcNAc branch on α1,3 Man (Oguri et al. 1997). GnT-V transfers β1,6-linked GlcNAc onto α1,6 Man (Shoreibah et al. 1993). GnT-III catalyzes the transfer of β1,4-linked GlcNAc onto a β-linked core Man, and the produced GlcNAc is called a bisecting GlcNAc (Nishikawa et al. 1992). The other type of core *N*-glycan modification is α1,6-fucose on GlcNAc at the reducing end. α1,6-fucosyltransferase (FUT8) catalyzes this reaction, and the structure is called the core fucose (Uozumi et al. 1996; Yanagidani et al. 1997). In vitro enzymatic analyses revealed the requirement of β1,2-linked GlcNAc on α1,3 Man, indicating that FUT8 requires the prior action of GnT-I (Wilson et al. 1976). GlcNAc residues produced by GnT enzymes are further extended by Gal and Sia residues. Gal is transferred by β1,4- or β1,3-galactosyltransferases, whereas Sia is added by α2,6- or α2,3-sialyltransferases (Stanley et al. 2015). In some glycoproteins, LacNAc units (Gal β1,4-GlcNAc) are repeatedly added to produce poly-LacNAc structures. Poly-LacNAc is thought to be involved in cancer malignancy because of its regulatory role in cell proliferation (Partridge et al. 2004). *N*-Glycans on certain glycoproteins harbor GalNAc instead of Gal, forming the LacdiNAc structure. In addition, *N*-glycans can be further modified with Fuc, GlcA, Sia, and sulfate residues to form various terminal epitopes, and their syntheses are highly dependent on cell type and glycoprotein.

1.3.2 *N*-Glycosylation-Dependent Trafficking of Lysosomal Enzymes

The major lysosomal enzymes involved in the degradation of proteins, lipids, and carbohydrates should be efficiently targeted to lysosomes instead of secretion or targeting to other compartments. Man-6-P, a phosphorylated *N*-glycan structure, is a common tag for lysosomal targeting of these enzymes (Distler et al. 1979; Natowicz et al. 1979) and is generated on oligomannose or hybrid-type *N*-glycans of lysosomal enzymes (Castonguay et al. 2011). Phosphorylation involves two sequential steps: transfer of GlcNAc-1-P and subsequent removal of the GlcNAc moiety. GlcNAc-phosphotransferase, which catalyzes the first step (Lang et al. 1986), is a Golgi-resident large hexameric complex composed of two α, two β, and two γ subunits (Coutinho et al. 2012). The α and β subunits are encoded by a single *GNPTAB* gene and translated as a precursor protein comprising 1256 amino acids (Tiede et al. 2005). Site-1 protease cleaves the precursor, generating mature α and β subunits (Marschner et al. 2011), and this reaction is essential for the enzyme to acquire activity. The γ subunit encoded by the *GNPTG* gene is a soluble protein and forms a homodimer via a disulfide bond (Raas-Rothschild et al. 2000). Although the α and β subunits are the catalytic subunits, the γ subunit is also required for enzymatic activity. The second step is mediated by *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, also known as uncovering enzyme (UCE) (Kornfeld et al. 1999). UCE undergoes a proteolytic cleavage at the trans-Golgi network (TGN) by furin for activation, and the active form of UCE catalyzes the removal of GlcNAc from the GlcNAc-P-Man diester to give Man-6-P (Do et al. 2002).

Man-6-P is recognized by two receptors, cation-independent or cation-dependent Man-6-P receptors (CI-MPR or CD-MPR, respectively). They are referred to as P-type lectins because of their ability to bind to the phosphate (Castonguay et al. 2011). CD-MPR consists of one MRH domain and forms a homodimer. CI-MPR has 15 MRH domains, and the third and the ninth domains contain Man-6-P binding sites, whereas the fifth domain has a binding site for a phosphodiester (Man-6-P-GlcNAc) (Tong et al. 1989; Reddy et al. 2004). The third and the ninth domains of CI-MPR have a similar affinity toward Man-6-P, suggesting their functional redundancy (Tong and Kornfeld 1989). The unique features of CI-MPR for binding to Man-6-P-GlcNAc could also contribute to the trafficking of lysosomal enzymes independent of UCE, as UCE-deficient mice show normal growth and no histological abnormalities (Boonen et al. 2009). MPRs bind to Man-6-P in a pH-dependent manner as observed for other lectins, as described above. MPRs preferentially bind to Man-6-P at pH 6.5 to 6.7 (typical pH in the TGN), whereas they dissociate from their ligands at pH 6 (late endosomal pH). MPRs are constitutively cycled between the TGN and the late endosomes via clathrin- and retromer-dependent manners, respectively (van Meel and Klumperman 2008).

1.4 Biological Roles of Branched *N*-Glycans and Corresponding Glycosyltransferases

As described above, mature *N*-glycans have diverse branched structures. In this section, we describe the physiological and pathological significance of the branched structures of *N*-glycans such as the core fucose, bisecting GlcNAc, β1,4-GlcNAc branch, and β1,6-GlcNAc branch.

1.4.1 Core Fucosylation by FUT8

α1,6-Fuc modification on the innermost GlcNAc is catalyzed by FUT8 (Uozumi et al. 1996; Yanagidani et al. 1997). The core fucose structure is abundant in *N*-glycans and plays critical roles in various physiological and pathological conditions. FUT8 knockout (KO) mice show various severe phenotypes, including early death during postnatal development, growth retardation, lung emphysema, T-cell dysfunctions, and

schizophrenia-like behavioral abnormality (Wang et al. 2005; Gao et al. 2012; Fukuda et al. 2011; Fujii et al. 2016). The dysfunction of growth factor receptors such as epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor receptor (VEGFR) likely contributes to these phenotypes (Wang et al. 2005; Wang et al. 2006). In addition to the spontaneous lung emphysema in FUT8 KO mice, a human congenital disorder of glycosylation patients with a genetic mutation of *FUT8* commonly shows respiratory defects (Ng et al. 2018; Ng et al. 2020), suggesting that FUT8 is implicated in the development of chronic obstructive pulmonary disease (COPD). FUT8 activity was also reported to be associated with the exacerbation of human COPD (Kamio et al. 2012).

Expression of FUT8 is upregulated in several cancers such as hepatoma, melanoma, lung, breast, and prostate cancers (Potapenko et al. 2010; Wang et al. 2014; Saldova et al. 2011; Agrawal et al. 2017; Cheng et al. 2016; Chen et al. 2013), suggesting that the core fucose is associated with cancer malignancy. Furthermore, core-fucosylated proteins can be used as biomarkers, exemplified by fucosylated alpha-fetoprotein (AFP), AFP-L3, being approved for early detection of liver cancer (Taketa et al. 1993; Aoyanagi et al. 2010). Regarding cancer therapy, it was demonstrated that loss of the core fucose of IgG *N*-glycan greatly enhances the activity of antibody-dependent cellular cytotoxicity by approximately 50-fold (Shields et al. 2002; Shinkawa et al. 2003). This illustrates the importance of the *N*-glycan structure, core fucose in particular, for antibody-based therapeutics. The development of small molecules to control *N*-glycan structures on IgG has progressed (Chang et al. 2019).

FUT8 is a type-II transmembrane protein similar to other Golgi-localized glycosyltransferases but uniquely has an src homology 3 (SH3) domain in its luminal region (Ihara et al. 2007). SH3 domains are typically found in cytosolic proteins to facilitate protein-protein interactions (Zafra-Ruano and Luque 2012), and the function of the FUT8 SH3 domain has not been elucidated until recently. Two independent groups recently reported the crystal structures of the FUT8 catalytic domain together with an *N*-glycan substrate (Garcia-Garcia et al. 2020; Jarva et al. 2020). These structures clearly demonstrated that FUT8 SH3 is associated with the *N*-glycan acceptor, suggesting the requirement of the SH3 domain for FUT8 activity. We and other groups have biochemically supported these findings (Ihara et al. 2020; Tomida et al. 2020). We showed that deletion or point mutation of the SH3 domain resulted in nearly complete loss of activity, indicating that the FUT8 SH3 domain is required for *N*-glycan substrate recognition (Tomida et al. 2020). Moreover, we surprisingly found that FUT8 is partially localized at the cell surface and deletion of the SH3 domain reduced the cell surface level of FUT8 (Tomida et al. 2020). This indicates that FUT8 localization is uniquely regulated in an SH3-dependent manner. We also identified ribophorin I, an OST subunit, as a FUT8 interaction partner (Tomida et al. 2020), and ribophorin I binds to FUT8 in an SH3-dependent manner. As depletion of ribophorin I reduced FUT8 activity, ribophorin I is a positive regulator of FUT8 activity (Tomida et al. 2020). It will be important to clarify how ribophorin I regulates FUT8 activity and whether ribophorin I is involved in FUT8 localization at the cell surface.

1.4.2 Bisecting GlcNAc by GnT-III

GnT-III catalyzes the transfer of GlcNAc to β-Man via a β1,4-linkage to generate bisecting GlcNAc (Nishikawa et al. 1992). Bisecting GlcNAc uniquely inhibits the activities of many glycosyltransferases, including GnTs, FUTs, and sialyltransferases (Schachter 1986; Gu et al. 1993; Nakano et al. 2019). Mechanistically, the presence of bisecting GlcNAc causes steric hindrance against the catalytic pocket of GnT-V (Nagae et al. 2018). Furthermore, molecular dynamics simulations revealed that bisecting GlcNAc alters the overall structure of *N*-glycans to a back-fold conformation, leading to reduced

affinity with other glycosyltransferases (Nakano et al. 2019). Therefore, GnT-III has regulatory activities of *N*-glycan maturation, and aberrant expression of GnT-III causes a dramatic change to *N*-glycan structures in cells.

The expression of GnT-III is tissue-specific with the highest expression in the brain and kidney (Miyoshi et al. 1997) and aberrantly induced in some cancer cells (Miyoshi et al. 1993). GnT-III-KO mice showed a slower proliferation of cancer cells in a diethylnitrosamine-induced liver cancer model (Yang et al. 2003). In contrast, another report using a GnT-III-overexpressing B16 melanoma cell line showed reduced metastasis in lung tissue (Yoshimura et al. 1995). These results suggest that bisecting GlcNAc regulates cancer malignancy and is probably dependent on cancer cell types. E-cadherin was identified as a target glycoprotein of GnT-III involved in cancer phenotypes, and overexpression of GnT-III resulted in the prolonged retention of E-cadherin at the cell surface, leading to increased cell adhesion activity (Yoshimura et al. 1996).

The high expression of GnT-III in the brain suggests significant roles of bisecting GlcNAc in this tissue. Our group reported that GnT-III-KO mice crossed with Alzheimer's disease model mice showed reduced accumulation of amyloid-β in the brain and a significant improvement of Alzheimer's disease pathology, including an improvement in short-term memory (Kizuka et al. 2015; Kizuka et al. 2016). This phenotype was caused by abnormal lysosomal localization of β-secretase (BACE1), a key enzyme required for the production of amyloid-β (Kizuka et al. 2015). Although the precise mechanism for the abnormal localization of BACE1 is currently unknown, the regulation of bisecting GlcNAc levels is a therapeutic candidate for Alzheimer's disease. The physiological significance of GnT-III has not been elucidated.

The regulation of GnT-III gene expression is currently not understood well. A recent genome-wide association study of IgG *N*-glycosylation postulated the transcriptional regulation pathway of the *MGAT3* gene. The single-nucleotide polymorphism affecting IgG *N*-glycosylation in the *MGAT3* locus was found in a region bound by the transcription factor RUNX3 (Klaric et al. 2020). The involvement of RUNX3 in *MGAT3* transcription needs to be experimentally confirmed.

1.4.3 β1,4-GlcNAc Branch Produced by GnT-IV

GnT-IV catalyzes the transfer of GlcNAc to α1,3-Man via the β1,4-linkage (Oguri et al. 1997). The GnT-IV family is composed of four family members, GnT-IVa, GnT-IVb, GnT-IVc, and GnT-IVd (Nagae et al. 2020). GnT-IVa and IVb have similar catalytic activities, whereas the activities of GnT-IVc and GnT-IVd have not been detected. The expression pattern of GnT-IVa is restricted to the pancreas, small intestine, and colon (Yoshida et al. 1998), whereas GnT-IVb is ubiquitously distributed. GnT-IVa has a higher affinity for both donor and acceptor substrates when compared with that of GnT-IVb (Oguri et al. 2006). GnT-IVa-KO mice show type 2 diabetes-like phenotypes, including significant elevation of the blood glucose level, free fatty acids and triglycerides, and reduced insulin levels (Ohtsubo et al. 2005; Ohtsubo et al. 2011). In pancreatic β cells, the glucose transporter 2 (GLUT2) that senses the blood glucose level was identified as the target protein of GnT-IVa. The expression of GLUT2 on the surface of β cells decreased in GnT-IVa-KO mice, resulting in the loss of glucose sensitivity for production of insulin. In contrast, GnT-IVb-KO mice showed milder phenotypes than GnT-IVa-KO mice. Although GnT-IVa and GnT-IVb double-KO mice completely lost GnT-IV enzyme activity (Takamatsu et al. 2010), the double-KO mice displayed a similar phenotype to that of the GnT-IVa-KO mice. Therefore, the physiological significance of GnT-IV, particularly for GnT-IVb, GnT-IVc, and GnT-IVd, remains unresolved.

Although no enzymatic activity of mammalian GnT-IVc has been detected, the counterpart enzymes from fish and chicken clearly have enzymatic activity, catalyzing the transfer of GlcNAc to the α1,6 Man via a β1,4-linkage

(GnT-VI activity) (Sakamoto et al. 2000; Taguchi et al. 2000). In mammalian cells, activity and products of GnT-VI have not been confirmed, and therefore, unique penta-antennary *N*-glycans are expressed in limited species such as fish and chicken. GnT-IVd, also known as GnT1IP, was found to be a GnT-I inhibitor (thus GnT1IP is referred to as GnT-I inhibitory protein) (Huang and Stanley 2010). GnT-IVd specifically interacts with GnT-I but not with other GnTs via its luminal domain and inhibits its activity (Huang et al. 2015b). GnT-IVd is expressed mainly in testicular germ cells, and expression is regulated during spermatogenesis, suggesting that GnT-IVd is required for proper spermatogenesis.

1.4.4 β1,6-GlcNAc Branch Produced by GnT-V

GnT-V catalyzes the transfer of GlcNAc residues to α1,6 Man via a β1,6-linkage (Shoreibah et al. 1993). In contrast to other GnTs, GnT-V does not require divalent cations because GnT-V lacks the DXD motif present in most GT-A type glycosyltransferases. We recently solved the crystal structure of the catalytic domain of GnT-V with or without a bisubstrate-type inhibitor and showed that GnT-V adopts a GT-B fold, consistent with this protein lacking the DXD motif (Nagae et al. 2018). Our GnT-V structure clearly visualized the GlcNAcβ1,2-Manα1,6-Man unit as a model acceptor substrate and showed that the GlcNAc residue is sandwiched between two aromatic residues, Phe380 and Trp401 (Nagae et al. 2018). Docking simulations showed that non-substrate structures, such as GlcNAcβ1,2-Manα1,3-Man, galactosylated GlcNAc on α1,6 Man, and bisecting GlcNAc, all sterically clash with the catalytic groove, which is consistent with the acceptor specificity observed in biochemical assays (Brockhausen et al. 1988; Gu et al. 1993). GnT-V also shows site-specific preferences even in the same protein. For example, Asn554 of E-cadherin is modified by GnT-V, whereas Asn633 is not (Carvalho et al. 2016). The substrate-binding region of GnT-V, including Phe380 and Trp401, is buried inside the deep catalytic groove, raising the possibility that the deep groove strictly determines site specificity. A docking model suggested that *N*-glycan on Asn554 fits the groove, whereas *N*-glycan on Asn633 does not because of steric hindrance between GnT-V and E-cadherin. Therefore, the deep catalytic groove of GnT-V could strictly determine substrate specificity, including the *N*-glycan structure and *N*-glycosylation site.

GnT-V is well-known to be involved in cancer development and malignancy. The Ras-Raf-Ets2 signaling pathway drives transcription of the *MGAT5* gene (Kang et al. 1996; Buckhaults et al. 1997). Target proteins of GnT-V in cancer include cell surface receptors and cell adhesion molecules. The endocytosis of EGF receptors and TGF-β receptors, for example, is inhibited by the overproduction of β1,6-branches, promoting cell proliferation (Partridge et al. 2004). Galectins contribute to this effect through binding to the poly-LacNAc units synthesized on β1,6-GlcNAc branches. Additionally, cell adhesion is negatively regulated by GnT-V. For example, the β1,6-branch on the specific site of α5β1 integrin reduces its adhesion ability and intracellular signaling (Isaji et al. 2006). In addition, the β1,6-branch on N-cadherin is involved in reduced cell adhesion (Guo et al. 2009). Through these phenomena, GnT-V promotes cell migration and therefore cancer metastasis. Consistent with the cancer-promoting roles of GnT-V in cell experiments, cancer growth of a PyMT-induced model was reduced significantly in GnT-V-KO mice (Granovsky et al. 2000). Therefore, inhibition of GnT-V activity or expression can be a potential therapeutic for cancer, and the solved GnT-V structure should contribute to designing inhibitors that inhibit the catalytic activity of GnT-V.

The activity of GnT-V is also regulated at the protein level. GnT-V was shown to be proteolytically cleaved by the Golgi-localized protease, signal peptide peptidase-like 3 (SPPL3) (Voss et al. 2014; Kuhn et al. 2015). SPPL3 cleaves the C-terminal side of the transmembrane domain of GnT-V, and the cleaved form is secreted into the culture medium. SPPL3-KO cells showed increased expression of GnT-V and its product glycans in cells. Conversely, the cells reduced the

secretion of GnT-V into the culture medium. Therefore, regulation of SPPL3-dependent shedding of GnT-V might also be a pharmaceutical approach for GnT-V-targeted cancer therapy.

1.5 Concluding Remarks

In this chapter, we have provided an overview of the *N*-glycosylation pathway from the ER to the Golgi and described the roles of *N*-glycan branches. The complete *N*-glycosylation pathway has been almost fully identified by research conducted over the last 30 years. Detailed mechanisms describing the action of each glycosyltransferase, however, remain largely unresolved. Structural analyses of the enzymes are needed for this purpose. Moreover, the regulation of the activity and expression of glycosyltransferases remain poorly understood. Recent studies using super-resolution microscopy postulated the presence of a specialized zone in the Golgi for each glycosylation, indicating that the *N*-glycosylation pathway is more complex. We hope that these issues will be tackled and clarified in the next decade.

Compliance with Ethical Standards

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2 Mucin-Type *O*-GalNAc Glycosylation in Health and Disease

Ieva Bagdonaite, Emil M. H. Pallesen, Mathias I. Nielsen, Eric P. Bennett, and Hans H. Wandall

Abstract

Mucin-type GalNAc *O*-glycosylation is one of the most abundant and unique post-translational modifications. The combination of proteome-wide mapping of GalNAc *O*-glycosylation sites and genetic studies with knockout animals and genome-wide analyses in humans have been instrumental in our understanding of GalNAc *O*-glycosylation. Combined, such studies have revealed well-defined functions of *O*-glycans at single sites in proteins, including the regulation of pro-protein processing and proteolytic cleavage, as well as modulation of receptor functions and ligand binding. In addition to isolated *O*-glycans, multiple clustered *O*-glycans have an important function in mammalian biology by providing structural support and stability of mucins essential for protecting our inner epithelial surfaces, especially in the airways and gastrointestinal tract. Here the many *O*-glycans also provide binding sites for both endogenous and pathogen-derived carbohydrate-binding proteins regulating critical developmental programs and helping maintain epithelial homeostasis with commensal organisms. Finally, *O*-glycan changes have been identified in several diseases, most notably in cancer and inflammation, where the disease-specific changes can be used for glycan-targeted therapies. This chapter will review the biosynthesis, the biology, and the translational perspectives of GalNAc *O*-glycans.

Keywords

Glycans · GalNAc · Carbohydrate-binding proteins · Proprotein processing · Mucins · Cancer

2.1 Introduction

Almost 150 years ago, the founder of biochemistry E. Hoppe-Seyler, and doctor and biologist E. Eichwald first noted *O*-glycans on isolated proteins from human blood and other secretions primarily on large mucins (Brockhausen et al. 2009). Since then, we have learned how this diverse class of glycans, later known as mucin-type *O*-glycosylation or *O*-*N*-acetylgalactosamine (*O*-GalNAc) glycosylation, occurs not only as

The original version of this chapter was revised: Figure 2.1 was inadvertently published with some colors illustrating important chemical differences lost on the right side of the image which has been corrected now. The correction to this chapter is available at https://doi.org/10.1007/978-3-030-70115-4_18

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dense clusters on mucins but also as single sites on a large proportion of secreted and membrane-bound proteins. Indeed, we now know that *O*-GalNAc glycans (in the following just referred to as *O*-glycans) are involved in almost every aspect of biology, including cell–cell communication, cell adhesion, signal transduction, immune surveillance, and host–pathogen interactions (Varki 2017). In densely glycosylated proteins such as mucins, *O*-glycans support larger mucin domains that provide both an efficient barrier against and a habitat for pathogenic organisms (Johansson et al. 2011; Arike and Hansson 2016). This is very different from the function of single *O*-glycosylation sites. Here the existence of 20 polypeptide GalNAc-transferases (GalNAc-Ts) provides the possibility for flexible regulation of GalNAc addition to specific sites in proteins with potentially regulatory roles. For example, single glycosylation events co-regulate protein cleavage by proprotein convertases and control proteolytic cleavage by ADAM proteases (Goth et al. 2018), and in receptors such as low-density lipoprotein receptor (LDLR) and LDLR-related proteins (LRPs), single GalNAc-glycosylation sites support ligand binding (Wang et al. 2019). Finally, *O*-glycans, either in defined single sites or in clusters, may carry terminal ligands that mediate *cis* interactions between elements on the same cell and *trans* interactions between cells or different species (host–pathogen interactions) (Varki 2017). This omnipresence and multitude of functions that vary with glycan location and context make clarifying their biological roles a challenge (Bagdonaite et al. 2020; Dabelsteen et al. 2020).

Recently, technological advances in mass spectrometry and genetic engineering have allowed mapping of *O*-glycosylation sites on human and viral proteins (Steentoft et al. 2013, 2014; Bagdonaite et al. 2015), leading to important discoveries in defining *O*-glycan structures that mediate specific molecular interactions. In this chapter, we describe the *O*-GalNAc glycan structures and their biosynthesis, including their initiation and substrate selectivity by GalNAc-Ts and their elongation by extension and branching enzymes. We next discuss the current understanding of the biological roles of *O*-glycans and review findings from global mapping of *O*-glycan sites and lessons learned from cell models, knockout animals, and genome-wide association studies (GWAS). Furthermore, we briefly review the exploitation of GalNAc *O*-glycans in therapeutic applications. These applications include targeting a specific PSGL-1 *O*-glycan structure as an anti-inflammatory treatment and the potential use of cancer-associated *O*-glycans in cancer prognostics and diagnostics. Novel antibody-based cancer therapeutic applications, such as bispecific T-cell engagers (BiTEs) and chimeric antigen receptor T cells (CAR-Ts) targeting cancer associated *O*-glycans, are also discussed.

2.2 *O*-GalNAc Glycan Structures

Protein GalNAc-*O* glycosylation involves the sequential addition of monosaccharides to a growing oligosaccharide chain resulting in core extension, branching, elongation, and final capping of glycans (Fig. 2.1). The simplest *O*-glycan is a GalNAc residue linked to a serine or threonine residue, or less frequently to tyrosine. The short glycan is often referred to as the T nouvelle antigen, or Tn-antigen, indicating that the simple GalNAc *O*-glycan is immunogenic (Friedenreich 1930). The Tn-antigen can be elongated and branched to create eight distinct *O*-glycan core structures, of which cores 1–4 are the most common (Brockhausen 2006). Core-1 and core-8 structures are created from an initial GalNAc with a galactose residue added, core-3 and core-6 have an added *N*-acetylglucosamine (GlcNAc) residue, and the rare core-5 and core-7 structures have a GalNAc added (Brockhausen 2006). The most prevalent of these different core *O*-GalNAc-glycans contain core-1 (Galβ3GalNAc) or core-3 (GlcNAcβ3GalNAc) structures, which can be further branched by transfer of an additional GlcNAc residue to form core-2 and core-4, as depicted in Fig. 2.1.

Almost all cells express the core-1 structure, and especially mucins and circulatory glycoproteins contain high amounts (Brockhausen 2006).

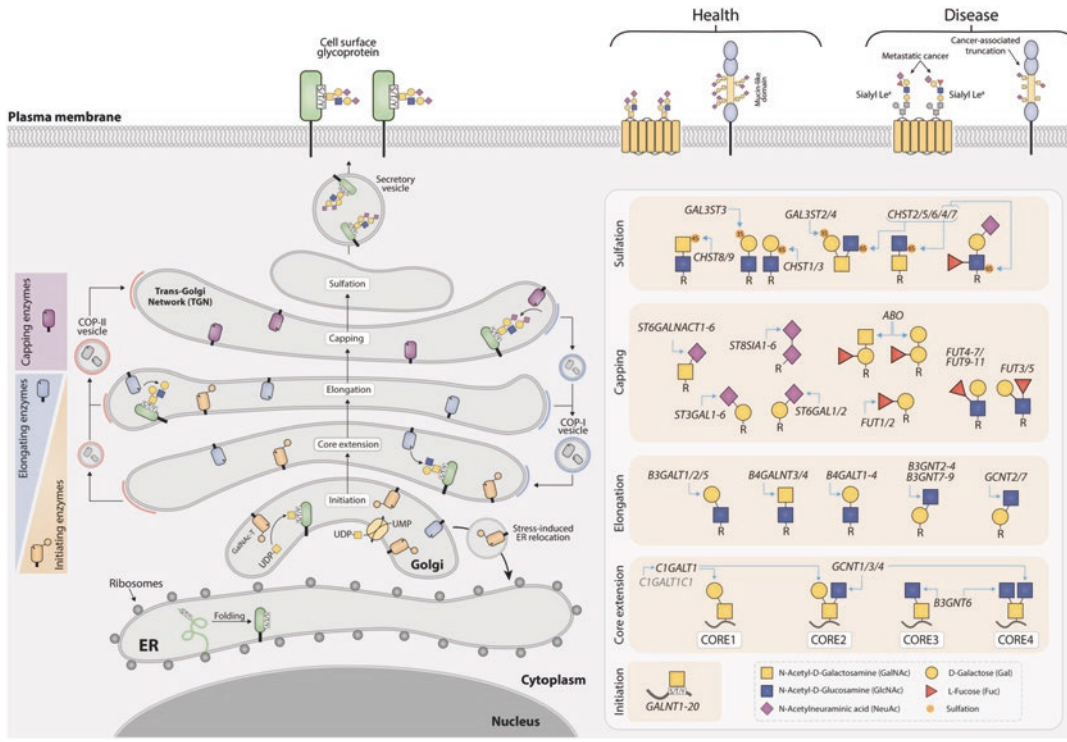

Fig. 2.1 *O*-GalNAc structures and biosynthesis
In healthy cells, the *O*-GalNAc biosynthesis pathway is restricted to the Golgi apparatus. A collection of different glycosyltransferases builds up the glycan structure as the carrier protein is transported through the different Golgi stacks and eventually ends up in secretory vesicles. The biosynthesis of the *O*-glycan structures can be divided into the following steps: 1) initiation, 2) core extension, 3) elongation, 4) capping, and 5) sulfation. The first initiation step is controlled by the members of the GalNAc-transferase enzyme family encoded by the *GALNT* genes. This family comprises 20 isoforms that catalyze the addition of a GalNAc monosaccharide to a threonine or serine (and in rare cases, tyrosine) residue in a newly synthesized protein. The subsequent addition of other sugars to the initial GalNAc will determine the type of core structure, with the four most common core structures depicted on the right. This panel also depicts the possible sugars that can be added to the extended *O*-glycan structure and the gene names for the respective enzymes. It is important to note that the enzymes are not limited to specific Golgi stacks. The gradient of initiating enzymes will decrease towards the Trans Golgi Network (TGN), while conversely, the gradient of branching and elongating enzymes will increase. The enzymes responsible for the final capping of the glycan structure are found in the outer stacks of the TGN. The resulting glycan structure can be affected by pathological conditions. For instance, malignant cancers are known for their lack of mature *O*-glycan structures on their surface proteins. Two other characteristic structures are the sialyl-Lewis-X and the sialyl-Lewis-A, which are upregulated in highly metastatic cancers

The core-2, core-3, and core-4 structures are found on proteins and secretions from mucous membranes, in particular in the gastrointestinal and respiratory epithelia, as well as in salivary glands (Brockhausen et al. 2009). The remaining core structures have a much more restricted expression and have thus far been found only in very select conditions and secretions. Among these is core-5 (GalNAcα1,3GalNAc), which has been observed in human meconium and occasionally in gastric and intestinal adenocarcinomas (Hounsell et al. 1985; Jin et al. 2017). Similarly, core-6 (GlcNAcβ1,6GalNAc) and core-8 (Galα1,3GalNAc) structures have been described only sporadically. The core-6 structure has been observed in human intestinal and ovarian cyst mucin and human meconium, whereas core-8 has been identified in human respiratory mucins (Brockhausen et al. 2009). In contrast, core-7 (GalNAcα1,6GalNAc) structures have been detected only in bovine submaxillary mucins (Brockhausen et al. 2009).

In some cells, the major core structures are elongated with glycans that are shared among multiple classes of carbohydrates (Fig. 2.1). Elongation primarily involves *N*-acetyllactosamine (LacNAc type 2 chain Galβ1-4GlcNAc), often in the form of repeating disaccharides (PolyLacNAc) and branches (Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6] Galβ1-4GlcNAc). The type 1 disaccharide LacNAc (Galβ1-3GlcNAc) and the disaccharide LacDiNAc (GalNAcβ1-4GlcNAc) are also found as terminal disaccharides on a common scaffold of LacNAc glycans. The capping step primarily involves terminal decoration of the oligosaccharide chains with fucose and *N*-acetylneuraminic acid (known as NeuAc) and is directed by the large sialyltransferase and fucosyltransferase families (Fig. 2.1) (Tsuji et al. 1996; Cummings 2009; Harduin-Lepers et al. 2001; Breton et al. 1998). In addition, blood type antigens, such as ABO and Lewis structures, can decorate *O*-glycans at their termini and serve as important recognition motifs for lectins often essential for biological functions such as extravasation of immune cells to sites of inflammation (Varki 2017).

2.3 Biosynthesis of GalNAc *O*-Glycans

Protein *O*-GalNAc glycosylation is a multi-step process in the secretory pathway, predominantly in the Golgi apparatus, that determines which proteins are glycosylated, where glycans are positioned in those proteins, and the structures located at the individual *O*-glycosylation sites (Stanley 2011). Similar to the other glycosylation types, *O*-GalNAc glycosylation is controlled by the repertoire and kinetics of the glycosyltransferases in the Golgi apparatus and their localization in specific compartments (Stanley 2011). In addition, the formation of homomeric or heteromeric enzyme complexes may also affect orchestration of the glycosylation process (Kellokumpu et al. 2016; Hassinen et al. 2010, 2011). Other factors contribute to the final *O*-glycosylation outcome for a given cell, including availability of activated sugar donors and cofactors such as divalent cations, specific chaperones, pH, substrate availability, competition with other glycosylation pathways, and possibly the speed of intracellular transport (Stanley 2011). In the following, we briefly describe the glycosyltransferases involved in the initiation and formation of *O*-GalNAc core structures.

2.3.1 Initiation of *O*-GalNAc Glycosylation

Initiation of *O*-GalNAc glycosylation is catalyzed by 20 homologous polypeptide GalNAc-Ts encoded in the human genome (Bennett et al. 2012; Ten Hagen et al. 2003). As noted, GalNAc-Ts transfer GalNAc from a UDP-GalNAc donor to an acceptor oxygen in an α1 linkage to primarily serine or threonine and more rarely to tyrosine residues (Bennett et al. 2012). The individual GalNAc-Ts have different but overlapping substrate specificities and are differentially expressed in cells and tissues (Wandall et al. 1997; Bennett et al. 2012; Ten Hagen et al. 2003). Based on the retained stereochemistry from the UDP-GalNAc donor in the synthesized Tn-antigen, GalNAc-Ts are grouped in the GT27 CaZy family (Lombard et al. 2014). Furthermore, phylogenetic analysis suggests classification of GalNAc-Ts into two major families (I and II), again divided into subfamilies with similar predictive functions (Ia–g and IIa-b) (Schwientek et al. 2002; Bennett et al. 2012). The functional similarity among subfamily members has in part been confirmed based on *in vitro* glycosylation of small synthetic peptides (Bennett et al. 1999b; Lavrsen et al. 2018; Schwientek et al. 2002; Kong et al. 2015). The common belief is that new GalNAc-Ts have evolved with new substrate preferences to allow glycosylation of the variety of different substrates that are *O*-glycosylated. This association would predict an increasing number of GalNAc-Ts through evolution, which has indeed been observed with 9 members in *C. elegans*, 14 members in *Drosophila sp.*, 21 members in fish, 19 members in mice, and 20 members in humans (Bennett et al. 2012; Famiglietti

et al. 2017). Interestingly, yeast and plants lack GalNAc-Ts. In yeast, *O*-glycosylation is instead based on mannosylation (Strahl-Bolsinger et al. 1999), and plants rely on a plant-specific glycosylation in which they convert proline residues to hydroxyproline, where arabinose residues are attached (Strasser 2012).

GalNAc-Ts are type II membrane proteins characterized by a short N-terminal cytoplasmic tail, a transmembrane domain, and a C-terminal domain comprising a stem region and a Golgi luminal catalytic domain connected by a flexible linker to a ricin-type lectin domain (Bennett et al. 2012). The many structural studies of human GalNAc-T isoenzymes (T1, T2, T3, T4, T7, T10) clearly show that the N-terminal catalytic domain adopts a typical GT-A fold formed by two adjoining Rossmann-like folds linked by a short flexible linker to a C-terminal ricin-like lectin domain (Fritz et al. 2004, 2006; de Las Rivas et al. 2018, 2020; Lira-Navarrete et al. 2015; Kubota et al. 2006). The lectin domain, present only in eukaryotic GalNAc-Ts, has a β-trefoil fold built from three repeat units (α, β, and γ) that can bind a GalNAc moiety (Tenno et al. 2002a, b; Hassan et al. 2000; Wandall et al. 2007; Pedersen et al. 2011a).

Initiation of GalNAc-type *O*-glycosylation is entirely a post-translational event, taking place after folding and subunit assembly are completed in the endoplasmic reticulum (ER). Thus, a considerable number of GalNAc-*O*-glycan substrates would be expected, and consistent with this, there is no clear global or isoform-specific consensus motif for GalNAc-Ts. The catalytic activity is the main driver for substrate selectivity and depends on coordination between the divalent cation (Mn^{2+}) in the DxH motif and the GT-A fold catalytic domain (Lairson et al. 2008). *In vitro* glycosylation results show that GalNAc-T specificity is primarily defined by the amino acids surrounding the glycosylated Ser/Thr/Tyr (positions -5 to +5), with varying preferences for proline in the -3 and -1 position observed for different isoforms (Gerken et al. 1997; 2006; Mohl et al. 2020; Kong et al. 2015; O'Connell and Tabak 1993; Nehrke et al. 1997).

Adding another level of complexity, GalNAc-Ts exert glycopeptide activity depending on or influenced by existing GalNAc residues in their acceptor substrate. The glycopeptide activities of the GalNAc-Ts are categorized into two classes, based on their short-range (or neighboring, 1–3 residues) and long-range (<17 residues) glycosylating capabilities (de Las Rivas et al. 2019). In this way, a few GalNAc-Ts, including GalNAc-T7 and GalNAc-T10, glycosylate only substrates with a pre-existing GalNAc residue on neighboring amino acids and are thus designated as short-range follow-up enzymes (Bennett et al. 1999a; Ten Hagen et al. 1999; Cheng et al. 2002). In addition to these molecules with specificity defined by the catalytic domain, more distant *O*-GalNAc residues (long range; <17 residues) guide the substrate specificity of most GalNAc-Ts. They do so through the C-terminal ricin-like lectin domain found in all GalNAc-Ts, except for GalNAc-T20 (Bennett et al. 2012; Tenno et al. 2002a, b; Hassan et al. 2000; Wandall et al. 2007; Pedersen et al. 2011a). The length of the linker between the lectin and catalytic domains is important for positioning the catalytic domain, providing long-range specificity of the isozymes (de Las Rivas et al. 2019).

Other structural differences can be used to subdivide the GalNAc-Ts, such as the orientation of the catalytic domain toward previous *O*-GalNAc glycans in either the C- or N-terminal orientation (Gerken et al. 2013; de Las Rivas et al. 2019). As noted, the catalytic domain, length of the flexible linker, lectin domains, and their orientation will account for the different glycosylation preferences, but there is some redundancy among isoenzymes (de Las Rivas et al. 2019). Indeed, most individual GalNAc-Ts have distinct but overlapping substrate specificities *in vitro* (Wandall et al. 1997; Bennett et al. 2012; de Las Rivas et al. 2019). This concept has partially been confirmed by mass spectrometry analysis of the total *O*-glycoproteomes from wild-type and cells knocked out for select *GALNT* genes, also emphasizing that individual GalNAc-Ts have distinct non-redundant functions in a relevant biological context (Schjoldager et al. 2012, 2015; Lavrsen et al. 2018; Bagdonaite et al. 2020). A prominent example is the select glycosylation of peptide linkers between class A

repeats of the LDLR family by GalNAc-T11 (Wang et al. 2018; Pedersen et al. 2014).

2.3.2 Formation of *O*-GalNAc Core Structures Is Tissue Dependent

The repertoire of GalNAc-Ts defines target proteins and where in those proteins *O*-glycans are localized, but the different core synthases, together with elongating and capping enzymes, define the final glycan structures. Most of the core synthases are differentially expressed depending on tissue and cell differentiation. Because they have either a limited set of close paralogs, or none at all, it is possible to predict glycan structures produced in a given cell based on the expression of the core synthases. The two main core structures, core-1 and core-3, are respectively catalyzed by the core-1 synthase (C1GALT1) and the core-3 synthase (B3GNT6), which respectively add a β-linked Gal or GlcNAc (Ju et al. 2002a; Ju and Cummings 2002; Iwai et al. 2002). Both synthases belong to the GT31 CaZy family but differ in their expression profiles. While the core-1 synthase is ubiquitously expressed, the core-3 synthase is primarily expressed in the healthy gastrointestinal tract with a predominant expression in the proximal part of the colon (Iwai et al. 2002; Ju and Cummings 2002; Ju et al. 2002b; Bergstrom et al. 2017; An et al. 2007). The core-1 synthase differs from the other core synthases by the requirement of the chaperone COSMC (C1GALT1 chaperone 1 (*C1GALT1C1*)) for proper folding and Golgi localization (Aryal et al. 2010, 2012). Thus, removal of either the core-1 synthase or its chaperone prevents synthesis of core-1–derived glycans (Ju and Cummings 2002). The two core-2 synthases, C2GnT1 and C2GnT3, convert core-1 glycans to core-2 glycans, whereas the core-4 synthase, C2GnT2, converts core-3 glycans to core-4 glycans (Stone et al. 2009) (Fig. 2.1). The three core synthases all belong to the GT14 CaZy family (Stone et al. 2009; Lombard et al. 2014) and are differentially expressed. Although the C2GnT1 synthase is ubiquitously expressed, the C2GnT3 (GCNT4) is selectively expressed in the immune system, liver, spleen, and the small intestine (Stone et al. 2009). The core-4 synthase, C2GnT2 (GCNT3), is co-expressed with the core-3 synthase and primarily present in the stomach and the proximal part of the colon (Stone et al. 2009; Bergstrom et al. 2017).

2.4 Biological Functions of *O*-GalNAc Glycosylation

2.4.1 Overview of *O*-Glycan Function

It can be challenging to tackle the biological roles of glycans because of their omnipresence and the multitude of different functions that can vary by the location and context of the individual glycan. There have been many attempts to classify glycans by function. Proposed categorizations have included arranging them by (1) structural and modulatory functions, (2) glycan–receptor interactions for adhesion and cell–cell communication, (3) interspecies functions such as host–pathogen interactions, and (4) molecular mimicry of host glycans by pathogens to avoid immune recognition (Varki 2017) (Fig. 2.2).

As mentioned above, the more clustered mucin-like *O*-glycans must be distinguished from single *O*-glycan sites. In densely glycosylated proteins such as mucins, *O*-glycans provide structural modifications of the protein backbone to protect and support larger mucin-like domains. Intestinally secreted gel-forming mucin MUC2 forms higher-order supramolecular structures to provide an efficient barrier against pathogenic organisms (Johansson et al. 2011). In this case, the *O*-glycans often function as part of the physical protection safeguarding a healthy host–pathogen interaction (Arike and Hansson 2016; Arike et al. 2017). In addition, the often highly glycosylated stem regions are important in securing correct protrusion of membrane-bound proteins with functional termini, such as PSGL-1 (Mcever and Cummings 1997; Shao et al. 2015; Jentoft 1990). Moreover, *O*-GalNAc glycans affect the

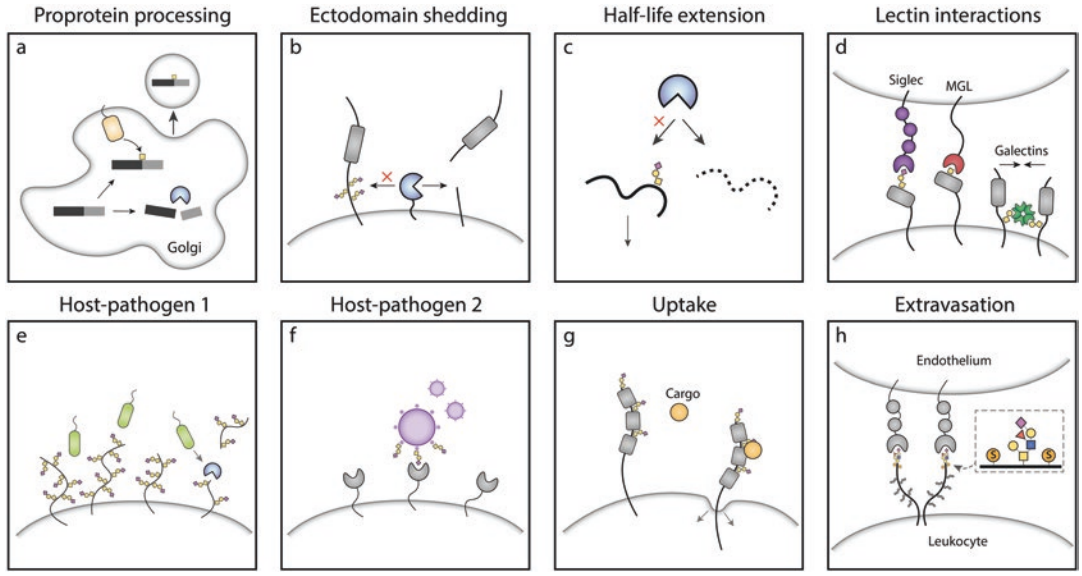

Fig. 2.2 Functions of *O*-glycans in biology
Cleavage protection is a general feature of *O*-glycans that has been described extensively. The biological consequences of this protection include the following: (**a**) shielding from proprotein convertases in the TGN, which in the case of FGF23 is essential for secretion of an active product; (**b**) glycosylation of stem regions in extracellular membrane proteins, which protects from ectodomain shedding by membrane resident proteases such as ADAM proteases and beta-secretase; and (**c**) glycosylation-mediated protease shielding of peptide hormones, enhancing their biostability in circulation. Specific *O*-glycan structures can also facilitate interactions with proteins with carbohydrate-recognition domains. These interactions include the following: (**d**) Siglec binding to terminal sialic acids, MGL binding to exposed GalNAc structures, and galectin-3 and galectin-8 interacting with beta-galactoside–containing *O*-glycan structures. *O*-glycans also have important roles in host–pathogen interactions: (**e**) the heavily *O*-glycosylated mucins form a protective barrier on mucosal surfaces, and some bacterial strains have evolved *O*-GalNAc–specific proteases that help them penetrate this protective barrier; and (**f**) more recently, *O*-glycans have also been found on viral surface proteins where, among other things, they help the virus escape host immune surveillance and interact with entry receptors. In some cases, the exact position or structure can also be critical for their function, with examples that include (**g**) GalNAc-T11, which has a unique ability to glycosylate linker regions between LDLR class A repeats found in cargo-uptake receptors, influencing the rate of uptake; and (**h**) the leukocyte extravasation process. Here, P-selectin on endothelial surfaces is dependent on a specific terminal core 2 sialyl-Lewis-X structure on leukocyte-bound PSGL-1. In addition, high-affinity binding is seen only when the core-2 structure is flanked by specific tyrosine sulfations

stability of several membranes and secreted proteins (Jentoft 1990). Especially, densely *O*-glycosylated regions in the stem protect proteins from proteolytic cleavage by secretase and ectodomain shedding (Jentoft 1990; Goth et al. 2015). This shedding is believed to affect the stability of, for example, LDLRs, VLDLRs, and LRPs, and alter the cellular uptake of LDL (Kingsley et al. 1986). The importance of dense glycosylation versus individual glycosylation sites, however, has not been evaluated in most of these studies.

The function of single *O*-glycosylation sites differs from that of the presumably non-regulated, clustered *O*-glycans. For single sites, the 20 GalNAc-Ts offer the possibility for regulatory roles of site-specific glycosylation. For example, single glycosylation events co-regulate protein cleavage by proprotein convertases as well as proteolytic cleavage and shedding of ectodomains by ADAM proteases (Goth et al. 2018; Schjoldager et al. 2011). Single-site effects have also been defined in receptors like LDLRs and LRPs, which are important for supporting ligand binding (Pedersen et al. 2014; Wang et al. 2018), and in contrast to the often-protective and stabilizing effects of the *O*-glycans localized in the stem regions (Kingsley et al. 1986). Furthermore,

several studies have shown that *O*-glycans affect cell–cell and cell–matrix interactions, influencing tissue formation and differentiation (Tian et al. 2012; Bagdonaite et al. 2020; Dabelsteen et al. 2020; Xia et al. 2004; Radhakrishnan et al. 2014; Zhang et al. 2008; Lavrsen et al. 2018). Indeed, a large proportion of surface receptors and extracellular matrix (ECM) proteins are decorated with multiple GalNAc *O*-glycosylation sites (Radhakrishnan et al. 2014; Dabelsteen et al. 2020), although we have limited understanding of how glycans affect adhesive properties.

Finally, *O*-glycans, either in defined sites or as clusters, may serve as carriers of terminal ligands such as blood type antigens (e.g., sialyl-Lewis-X) (Mcever 1997). These terminal structures mediate both *cis* interactions between elements on the same cell and *trans* interactions between different cells and even between different species (host–pathogen interactions) (Varki 2017). For example, programmed changes in *O*-glycan core structures play a role in B- and T-cell maturation and homing (Earl et al. 2010; Giovannone et al. 2018; Hernandez et al. 2007). Moreover, *O*-glycan terminal motifs on some immune cell subtypes can engage specific sialic acid-binding immunoglobulin-type lectins and induce tolerance to self-antigens (Duan and Paulson 2020). Concomitantly, similar structures can be recognized by bacterial and viral adhesins. During evolutionary host–pathogen adaptation, bacteria and viruses have evolved to use *O*-linked glycans for binding or entering cells (Varki 2017). One way of binding to cells is through directly engaging with terminal structures. Examples include *H. pylori* interaction with histo-blood group antigen carriers on gastric epithelia or that of *Staphylococci* with *O*-glycan determinants on oral mucins as well as GP1Balpha on platelets (Bensing et al. 2016). Another strategy used by bacteria is the molecular evolution of *O*-glycoprotein selective proteases that break down specific mucins to enable the pathogen to reach the target cells (Malaker et al. 2019). Viral pathogens have also evolved to use *O*-glycans in different ways. Some heavily *O*-glycosylated enveloped viruses acquire host-like glycosylation patterns by hijacking the host secretory pathway, and use the glycans as entry mediators by engaging, for example, MGL on dendritic cells as seen for Ebola virus, or, in the case of gammaherpesviruses, create a physical shield protecting immunodominant protein epitopes (Bagdonaite et al. 2018; Bagdonaite and Wandall 2018).

Clearly, the possible functions of GalNAc-*O*-glycans are overwhelming, and examining them all will be instrumental when mapping the location of individual *O*-glycans. Doing so could make it possible to interrogate the functions of glycans in the individual proteins. In this context, technological advances in mass spectrometry and genetic engineering have allowed for mapping *O*-glycosylation sites on human and viral proteins, leading to important discoveries related to how *O*-glycan structures mediate distinct molecular interactions (Steentoft et al. 2014).

2.4.2 Mapping the *O*-GalNAc-Glycoproteome

Until recently, only limited knowledge was available of where and which proteins carried *O*-GalNAc glycosylation, and was primarily based on glycan microarrays and analyses of glycan-binding proteins such as lectins and antibodies. The results have forwarded our understanding of *O*-GalNAc glycosylation but yielded only limited positional and structural information. A big step forward came with the proteome-wide mapping of *O*-GalNAc glycosylation sites. Several mass spectrometry-based strategies have been applied, including modifications of the endogenous glycan structures through chemical labeling (IsoTaG), lectin enrichment of structures derived from native or glycoengineered cells (SimpleCells), or *O*-glycan–specific endopeptidase treatment (the ExoO method) (Steentoft et al. 2011, 2013; King et al. 2017; Woo et al. 2017; Yang et al. 2018). With lectin-enrichment strategies, more than 15,000 sites have now been identified. Although the occupancy and relevance of all of these sites are unknown, the collective results demonstrate that *O*-GalNAc glycans modify more than 80% of

the proteins passing through the secretory pathway (Steentoft et al. 2013).

Analysis of the *O*-GalNAc glycoproteome has also allowed for unbiased identification of the protein features associated with *O*-GalNAc glycosylation (King et al. 2017). Enrichment of *O*-GalNAc glycosylation in mucin-like domains has been confirmed, as has enrichment within tandem-repeat regions, showing the anticipated preference for glycosylation of threonine over serine residues (King et al. 2017). *O*-GalNAc glycosylation is also enriched in serpin-reactive center loops, helix/coiled regions, the extracellular termini of multi-span proteins, the von Willebrand factor type A domain (found in, for example, β integrins), fibronectin type III domains, and protein stem, or linker regions (King et al. 2017). More rare domain structures include LDLR class A, thioredoxin, the EF-hand, Ig-like, sushi, and domains involved in ER/Golgi trafficking (King et al. 2017). Correlation of *O*-GalNAc glycosylation sites (±15 amino acids) with other annotated post-translational modifications and motifs has yielded a few well-known associations, such as proteolytic cleavage sites, along with some new ones, such as tyrosine sulfation and extracellular phosphorylation. The correlation with proteolytic cleavage sites also covers proprotein processing and extracellular ectodomain shedding, as found within the processed part of propeptides (Madsen et al. 2020; Hansen et al. 2019; King et al. 2017). This enrichment is in accordance with findings at the single protein level, where *O*-GalNAc glycosylation affects the proteolytic cleavage catalyzed by proprotein convertases and metalloproteases (Goth et al. 2018; Schjoldager et al. 2011).

O-GalNAc site mapping has thus expanded the glycobiology field from demonstrating the functional contribution of a few isolated sites to a broader appreciation of the generic properties of *O*-GalNAc glycosylation. What remains is the daunting task of assigning functions to each site, given the large and growing number of sites that have been identified. In this quest, several groups have used differential *O*-glycoproteomics to map isozyme-specific glycosylation sites in cells lacking individual GalNAc-Ts (Hintze et al. 2018; Schjoldager et al. 2012, 2015; Lavrsen et al. 2018; Bagdonaite et al. 2020). The major aim is now to understand the occupancy of the individual glycosylation sites, to map the sites and structures simultaneously, and to gain an understanding of their dynamic regulation.

2.4.3 *O*-glycans Regulate Protein Proteolysis

As described above, a consistent theme is regulation of protein proteolysis by site-specific *O*-glycosylation, with glycosylation of individual sites decreasing (or, more rarely, increasing) sensitivity to proteolysis (Goth et al. 2018; Schjoldager et al. 2011; King et al. 2018). The most well-documented example, among many, is the importance of site-specific *O*-glycosylation for regulating the circulating peptide hormone fibroblast growth factor-23 (FGF23) (Kato et al. 2006; Topaz et al. 2004). Osteocytes secrete FGF23, which acts on the kidneys to increase phosphate secretion. Proprotein convertases process FGF23 at a subtilisin-like proprotein convertase site (^{176}RHTR^{179}↓SAE^{182}), generating inactive N- and C-terminal fragments (Bergwitz and Juppner 2010). GalNAc-T3 glycosylates the cleavage site, which sits near a specific *O*-glycan (T178) that blocks proprotein processing. Disruptive mutations in GalNAc-T3 are linked to the metabolic disorder familial tumoral calcinosis (Kato et al. 2006), and glycosylation failure leaves FGF23 more susceptible to proteolysis and thus non-functional. The net result is decreased FGF23 signaling and failure to regulate phosphate excretion. In addition to an effect on FGF23, *O*-glycosylation also influences the small natriuretic peptides, including pro-brain natriuretic peptide (pro-BNP) and atrial natriuretic peptide (ANP), which both increase natriuresis. The *O*-glycan in pro-BNP is located close to the furin cleavage site, affecting cleavage and release of active BNP (Schellenberger et al. 2006; Nakagawa et al. 2017; Schjoldager et al. 2011). Moreover, change in *O*-glycosylation possibly affects BNP concentration in heart failure creating an possible link to the clinic (Costello-

Boerrigter et al. 2013). *O*-glycosylation of ANP seems to primarily shield it from proteolytic degradation in circulation while simultaneously inhibiting interaction with its receptor. The longer circulation time compensates for this inhibitory effect and results in a net increase in functionality (Hansen et al. 2019).

An additional relevant example is protection and prevention of autoactivation of plasma proteases (Bjorkqvist et al. 2013). Defective *O*-glycosylation of plasma protease factor XII, shown to be *O*-glycosylated *in vivo* (King et al. 2018), increases contact-mediated autoactivation of zymogen FXII, resulting in excessive activation of the bradykinin-forming kallikrein–kinin pathway that underlies hereditary angioedema type III, a rare inherited swelling disorder (Bjorkqvist et al. 2015). Other examples include the regulated proteolysis of the γ-melanocyte-stimulating hormone and β-endorphin from pro-opiomelanocortin (Birch et al. 1991). Yet other examples include glycosylation of TNFα, affecting its release by ADAM-17, metalloprotease cleavage of beta1 adrenergic receptor (Goth et al. 2017), and possibly the release of functional insulin-like growth factor-II (Hizuka et al. 1998).

Another important area, that seems tightly regulated by *O*-glycosylation is the protease network that regulates lipid metabolism, which in particular is affected by GalNAc-T2. This will be described in more detail below. However, here we should mention that site-specific glycosylation obviously affects many different functions simultaneously it is difficult to predict the physiological consequences of the loss of individual enzymes in whole organisms, yet alone, in individual cells. In an attempt to analyze the global effect of site-specific *O*-glycosylation on select protease networks, a positional proteomic strategy (terminal amine isotopic labelling of substrates (TAILS) was recently used (King et al. 2018). Surprisingly, this study demonstrated that the global loss of *O*-glycosylation governed by GalNAc-T2, in contrast to what was expected, decreased the cleavage of multiple substrates, most likely due to an effect on protease inhibitors and other key regulators of the proteolytic network (King et al. 2018).

2.4.4 Lessons Learned from Genetic Loss of Glycosyltransferases Involved in *O*-GalNAc-Glycosylation

2.4.4.1 *O*-GalNAc Initiation

Genetic loss of individual GalNAc-Ts has been an important step forward in understanding how site-specific *O*-GalNAc glycosylation influences biology. Loss or downregulation of individual GalNAc-T homologues in *D. melanogaster* using RNA interference demonstrated that the individual enzymes are essential for viability in some cases, whereas others are non-essential and exert only tissue- or maybe cell-specific effects (Tian and Ten Hagen 2006; Tran et al. 2012; Zhang and Ten Hagen 2010; Ten Hagen et al. 2009). A prominent example of cell-specific effects is how a lack of pgant3 affects the secretion of an integrin ligand involved in intercellular adhesion, and lack of pgant5 influences proper gut acidification (Zhang et al. 2010; Tran et al. 2012). Many knockout mouse models have been generated, including the elimination of GalNAc-T1, -T2, -T3, -T4, -T5, -T8, -T10, -T13, and -T14 (Bennett et al. 2012). None of the GalNAc-Ts were essential for viability, but obvious phenotypes were observed in the knockout of GalNAc-T1, -T2, and -T3 animals (Tian et al. 2015, 2019; Block et al. 2012; Tenno et al. 2007; Khetarpal et al. 2016; Ichikawa et al. 2009; Bennett et al. 2012). The relevance of the observed phenotypes in human biology, at least for GalNAc-T2 and GalNAc-T3, became obvious with results from GWAS and identification of patients deficient in GalNAc-T2 and -T3 (Teslovich et al. 2010; Zilmer et al. 2020; Topaz et al. 2004). Below, we briefly review the main lessons learned from genetic loss of a few select GalNAc-Ts and the major relevance for *O*-GalNAc-glycan biosynthesis.

2.4.4.2 GalNAc-T1 Potentially Affects Hemostasis and Intracellular Protein Sorting

Humans with deficiency in GalNAc-T1 activity have not yet been identified. However, generation of a murine model deficient in this GalNAc-T

showed that multiple biological processes depend on GalNAc-T1 (Tenno et al. 2007). This includes cardiac development, hemostasis, immune cell homing, and the ECM composition (Tenno et al. 2007; Tian et al. 2015; Block et al. 2012). In the hemostatic system, a lack of GalNAc-T1 leads to defective coagulation because of reduced plasma levels of blood coagulation factors (V, VII, VIII, IX, X, and XII) (Tenno et al. 2007). In this context, it is interesting that mapping of site-specific glycosylation in the hemostatic system identified multiple *O*-GalNAc sites in coagulation factors (King et al. 2017). Based on the murine phenotype, it is therefore possible that GalNAc-T1 is responsible for the initiation of some of these glycosylation sites. In support of this inference, GalNAc-T1-deficient mice also show reduced leukocyte homing resulting from reduced neutrophil membrane expression of E- and P-selectin ligands and decreased ligands for L-selectin on endothelial cells of peripheral lymph nodes (Tenno et al. 2007; Block et al. 2012).

Results of several murine studies also suggest a role for GalNAc-T1 in ECM deposition, as observed by phenotypic traits in the submandibular epithelium and the heart valves in models lacking GALNT1 (Tian et al. 2012, 2015). In the submandibular epithelium, the expression of basal membrane components such as laminin and collagen IV was decreased in *Galnt1* null mice (Tian et al. 2012). The observed effects on heart development correlated with an altered ECM composition, likely arising from altered metalloproteinase levels (Tian et al. 2015). Despite similarities, the GalNAc-T1 phenotypes also differed in several ways between the submandibular epithelium and the heart. For example, ER stress was found in the submandibular epithelium but not the heart, whereas the heart tissue showed increased proliferation, but the submandibular glands did not (Tian et al. 2012, 2015). This divergence in effect emphasizes that GalNAc-T1 has both contextual and general functions. In models of human epidermis, the loss of GalNAc-T1 induced morphological changes and changes in intracellular transport, with a number of cargo and transport proteins specifically *O*-glycosylated by GalNAc-T1 (Bagdonaite et al. 2020). This effect could suggest that changes in ECM composition in GalNAc-T1-deficient tissues may relate at least in part to the effects on intracellular trafficking (Bagdonaite et al. 2020).

2.4.4.3 Humans Lacking GalNAc-T2 Activity Show a Complex Phenotype

For more than 10 years, GWAS have associated GalNAc-T2 with lipid metabolism (Teslovich et al. 2010; Kathiresan et al. 2008). Recently, absent GalNAc-T2 activity has been identified in humans in association with a complex phenotype of dyslipidemia and thus altered high-density lipoprotein cholesterol and triglyceride levels (Khetarpal et al. 2016). Mice, rats, and cynomolgus monkeys also show this altered lipid metabolism with GalNAc-T2 deficiency (Khetarpal et al. 2016). The mechanism underlying this phenotype is unclear, but one proposed pathway involves a conserved network of GalNAc-T2 substrates that negatively regulate lipid metabolism (Khetarpal et al. 2016; King et al. 2018). People with GalNAc-T2 deficiency experience other complications as well. Exome sequencing of consanguineous families with neurodevelopmental disorders has revealed homozygous truncated variants of *GALNT2* among the candidate genes (Reuter et al. 2017), and clinical and molecular phenotypes of several patients with GalNAc-T2 deficiency have recently been described (Zilmer et al. 2020). Nevertheless, organ-specific mapping of GalNAc-T2 sites is incomplete and represents an arduous task, given the widespread tissue expression (White et al. 1995).

In vitro mapping and analysis of GalNAc-T2-deficient rodents have revealed the importance of GalNAc-T2 not only for lipid effects but also for the glycosylation of the β1-adrenergic receptor and TNFα (Goth et al. 2015, 2017). In both proteins, GalNAc-T2 glycosylation protects the substrates against premature cleavage by endogenous proteases. In case of the β1-adrenergic receptor, the glycosylation causes reduced receptor activity because of increased cleavage and deactivation (Goth et al. 2017; Park et al. 2017). In contrast, a lack of GalNAc-T2 leads to increased

TNFα activity, and the underlying mechanism is again a glycan-dependent protection of TNFα from cleavage and thus from ectodomain shedding associated with increased activity (Goth et al. 2015). Another function of GalNAc-T2 has been described in human keratinocytes, where loss of GalNAc-T2 function results in decreased cell–matrix adhesion, possibly due to altered site-specific *O*-glycosylation of a number of adhesion proteins selectively glycosylated by this isoform (Bagdonaite et al. 2020).

2.4.4.4 Humans Lacking GalNAc-T3 Activity Develop Familial Tumoral Calcinosis

Humans without GalNAc-T3 activity develop CDG hyperphosphatemic familial tumoral calcinosis because of decreased FGF23 signaling (Topaz et al. 2004). This condition was one of the first examples of the interplay between *O*-GalNAc glycosylation and proteolytic cleavage (Kato et al. 2006), later found to be a generic function. Maintaining FGF23 homeostasis requires a protective GalNAc-T3-catalyzed glycosylation to prevent proteolytic cleavage of FGF23. Thus, humans lacking GalNAc-T3 activity have decreased circulatory levels of non-cleaved bioactive FGF23. The result is increased phosphate reabsorption through the failure to suppress the NaPi-2a and NaPi-2c cotransporters in the proximal kidney tubules, leading to systemic hyperphosphatemia and cutaneous dermal mineralization (Chefetz and Sprecher 2009; Ichikawa et al. 2010; Gattineni et al. 2009). A mouse model of GalNAc-T3 deficiency that reflects the human syndrome (Ichikawa et al. 2009) also shows decreased levels of non-cleaved FGF23, but without the characteristic calcinosis (Ichikawa et al. 2009). Of interest, the co-regulation of proprotein convertase-mediated processing of FGF23 by GalNAc-T3 is subject to tight regulation through a feedback loop that involves control of *GALNT3* expression through blood phosphate levels, the fibroblast growth factor receptor, and the ERK signaling pathway (Takashi et al. 2019). GalNAc-T3 deficiency in the murine model has also been suggested to alter oral microbiome composition, likely because of diminished glycosylation of the salivary-secreted mucin Muc10 (Peluso et al. 2020). Furthermore, our results in an isolated epidermal organoid model suggest involvement of GalNAc-T3 in the timely induction of skin differentiation (Bagdonaite et al. 2020). These distinct roles for GalNAc-T3 in various tissues emphasize the multiple functions of this almost ubiquitous enzyme, likely dictated by organ-specific substrate repertoires.

2.4.4.5 Biological Functions for the Remaining GalNAc-Ts Are Primarily from GWAS and *In vitro* Studies

In humans, rare non-synonymous single-nucleotide variations have been identified in other GalNAc-Ts that do not result in enzymatic deficiency (Hansen et al. 2015; Joshi et al. 2018). GWAS have identified associations between human diseases and GalNAc-Ts. In addition to the association of *GALNT2* and changes in blood lipids, several other disease associations have been found, including between *GALNT4* variants and lower risk of acute coronary disease (O'Halloran et al. 2009), *GALNT5* and hereditary multiple exostoses through interaction with *EXT2* (Simmons et al. 1999), and *GALNT10* and sex-divergent risk for atherosclerosis (Dong et al. 2015). Rare *GALNT11* variants have been linked to heterotaxia associated with congenital heart disease, possibly because of defective glycosylation of Notch1, which is important for cilia-type expression (Fakhro et al. 2011; O'Halloran et al. 1985; Boskovski et al. 2013). More importantly, GWAS have linked the locus for *GALNTL5* (GalNAc-T20) and *GALNT11* to chronic kidney decline (Gorski et al. 2015), which might be explained by GalNAc-T11-specific *O*-glycosylation of LRP2 (Pedersen et al. 2014; Wang et al. 2019). LRP2, also known as megalin, is the major endocytic receptor in the proximal tubules of the kidney. In zebrafish and murine models, a lack of GalNAc-T11 compromises kidney function (Gorski et al. 2015; Tian et al. 2019). Similar to an effect on LRP2, GalNAc-T11 also glycosylates the ligand-binding region of LDLR, where GalNAc-T11-specific glycosylation

enhances the ligand affinity for the receptor (Pedersen et al. 2014, Wang et al. 2019). *GALNT12* variants additionally are linked to risk of colorectal cancer in a proportion of hereditary colorectal cancers (Clarke et al. 2012; Guda et al. 2009), and a similarly positive correlation has been described between *GALNT14* and hereditary neuroblastoma (de Mariano et al. 2015). GalNAc-T14 glycosylation and ligand binding to the death-receptor TRAIL have been identified, with a suggested but unverified role in inducing extrinsic apoptosis (Wagner et al. 2007). The apparent lack of phenotypes observed in mice deficient in the majority of GalNAc-T isozymes, combined with only a few mutations found in humans, suggests that more subtle phenotypes will be found in the murine models after more thorough examination exemplified by the continuously added effects found in mice and rats lacking GalNAc-T1, -T2, and -T3 expression, respectively.

2.4.5 The Biological Significance of the *O*-GalNAc Core Structures

In humans, systemic deficiencies have not been reported for any *O*-GalNAc core structures. Several diseases, however, are associated with changes in core-1 synthase activity or single nucleotide polymorphisms (SNPs) in the genes required for core-1 synthesis. For example, acquired somatic mutations cause the rare hematopoetic disorder Tn-syndrome (Berger 1999), and many types of cancers are associated with changes in *O*-glycan structures that we discuss below. In the following, we will briefly review the biological lessons from model organisms examining the main core structures.

2.4.5.1 Core-1–Derived *O*-GalNAc Glycans Serve Central Functions in Development and Maintenance of Homeostasis

Mice completely deficient in core-1 glycans die from hemorrhage during embryogenesis. For this reason, conditional, mosaic, and tissue-specific murine models have been developed to identify tissues requiring core-1 glycans (Song et al. 2017; Xia et al. 2004). With the ubiquitous expression of core-1-derived glycans, the murine models have highlighted their central roles in hematology, immunology, and epithelial biology. Tn-syndrome is a rare hematological disorder characterized by the expression of the Tn-antigen in all blood cell lineages, most likely because of the silencing of the core-1 synthase (Berger 1999). Clinically, patients with Tn-syndrome appear healthy but have moderate thrombocytopenia and leukopenia, and show some signs of hemolytic anemia (Berger 1999). Thrombocytopenia also occurs in mice with specific SNPs in *C1GALT1*, mice with a hematopoetic deficiency in core-1 synthase, and mice that are mosaic for *C1GALT1* (Alexander et al. 2006; Berger 1999; Kudo et al. 2013). The likeliest explanation is the lack of terminal megakaryocyte differentiation in these models (Kudo et al. 2013). In addition, there is increased clearance/phagocytosing of platelets by hepatic Kupffer cells, which terminal sialic acids normally inhibit (Li et al. 2017). Furthermore, endothelial *O*-GalNAc glycans are required for the interaction of terminal sialic acids on podoplanin with platelets (Pan et al. 2014). Thus, hemostasis depends on a complex network in which *O*-GalNAc glycans occur on many key players, including proteins involved in platelet aggregation, the coagulation cascade, and fibrinolysis (King et al. 2017; Sorensen et al. 2009; Wandall et al. 2012).

Core-1 glycans are also important in epithelial biology, as exemplified by functional changes in the kidneys, lymphatic vasculature, lung epithelial development, and intestinal epithelial homeostasis in the absence of such structures. In humans, GWAS have linked *C1GALT1* SNPs to nephropathies characterized by glomerulitis (Pirulli et al. 2009; He et al. 2012). Accordingly, nephropathies (glomerulitis) have been identified in murine models with either reduced core-1 synthase activity or with conditional post-natal or kidney-restricted core-1 synthase deficiency (Alexander et al. 2006; Song

et al. 2017). An identified change in podocyte foot projection morphology in these models possibly arises from altered podocalyxin signaling (Song et al. 2017; Stotter et al. 2020). Lymphatic vasculature integrity also depends on *O*-GalNAc glycans, with deficiency resulting in disorganized and blood-filled lymphatic vessels (Fu et al. 2008). Mice lacking either the lectin-like platelet activation receptor CLEC-2 or its endothelial ligand podoplanin show a similar phenotype, suggesting that podoplanin is a plausible carrier of crucial *O*-glycan chains (Osada et al. 2012; Fu et al. 2008). The conditional mouse models, however, lack terminal platelet differentiation and show potentially increased proteolytic processing of lymphatic endothelial podoplanin, so several mechanisms might produce the dysfunctional lymphatic vessels (Osada et al. 2012, Fu et al. 2008).

Other epithelial tissues that manifest the consequences of lacking core-1 glycans includes intestinal epithelia and oocytes. In the intestinal epithelia, core-1 glycans are vital for preventing spontaneous microbiota-dependent colitis (Jacobs et al. 2017; Kudelka et al. 2016), while lack of *O*-glycans in the oocyte hampers follicle homeostasis and oocyte development (Christensen et al. 2015; Fu et al. 2011). Thus, core-1 glycans are required for maintaining homeostasis within the hematological, immunological, and epithelial systems. Their ubiquitous expression indicates that they are probably equally important in other tissues, but complete tissue-specific model systems have not yet been established.

2.4.5.2 Core-2 Glycans Are Primarily Associated with Immune Regulation

Mice lacking both core-2 synthases proceed through embryogenesis, emphasizing that core-2 glycans, unlike core-1 glycans, are dispensable for embryogenesis (Stone et al. 2009). Deficiency in the ubiquitously expressed core-2 synthase C2GnT1 is associated with changes in a few tissues, together with an increased number of blood neutrophil granulocytes (i.e., primary neutrophilia) (Ellies et al. 1998). This effect in C2GnT1-deficient mice is believed to arise from reduced leukocyte homing, which in turn is trace to reduced biosynthesis of the common terminal structure SLe^x on core-2 glycans. This structure is important for binding to endothelial P-, E-, and L-selectins (Snapp et al. 2001; Ellies et al. 1998; Stone et al. 2009). The human relevance is further illustrated by primary neutrophilia in patients who lack the GDP-fucose transporter 1 required for the synthesis of SLe^x (Luhn et al. 2001). The absence of the other core-2 synthase, C2GnT3, results in only a minor increase in the number of neutrophils compared to C2GnT1 deficiency in a mouse model, reinforcing the fact that the function of core-2-derived glycans in leukocyte adhesion depends on C2GnT1 and not C2GnT3. In addition, in that study, the C2GnT3-deficient mice became more aggressive because of the decreased level of thyroxine in circulation (Stone et al. 2009).

2.4.5.3 Core-3– and Core-4–Derived *O*-GalNAc Glycans Regulate Intestinal Homeostasis

The core-3 synthase B3GnT6 and the core-4 synthase C2GnT2 are both primarily expressed in the gastrointestinal tract, and murine models lacking either of the two enzymes show disruption of the gastrointestinal mucosal barrier (An et al. 2007; Bergstrom et al. 2017; Stone et al. 2009). A potential mechanism is increased susceptibility of MUC2 to proteolysis by bacterial proteases. In mice with combined core-1 and core-3 synthase deficiency, elongated glycans are completely lacking, making the gastrointestinal mucins even more sensitive to proteolysis and the mice susceptible to chemical inducers of colitis and possibly carcinogenesis (Bergstrom et al. 2017). In the context of the protective effect of elongated and terminal glycan structures, the sialylation pattern change in patients with ulcerative colitis is relevant, possibly being caused by altered microbial composition and metabolism (Campbell et al. 2001b).

2.5 Mucin-Type GalNAc-*O*-Glycosylation in Acquired Diseases

2.5.1 Mucin-Type GalNAc-*O*-Glycosylation in Host–Pathogen Interactions

As mentioned above, one of the critical functions of cell surface glycans in vertebrates is to safeguard immune tolerance to self-antigens by engaging inhibitory receptors on lymphocytes (Duong et al. 2010; Perdicchio et al. 2016). In addition to defined endogenous functions, terminal glycan structures can serve as attractive targets for pathogens in accessing and entering target cells. A number of such structures can be found on both *N*-linked and *O*-linked glycans, as well as glycosphingolipids. For example, influenza viruses use terminal sialic acids, which are essential for the induction of tolerance, for attachment and subsequent infection of respiratory epithelial cells (Couceiro et al. 1993). Some human coronaviruses are proposed to depend on host sialic acids (Tortorici et al. 2019), and sialic acids on host *O*-glycans are thought to mediate the erythrocyte attachment of the malarial parasite *Plasmodium falciparum* (Jaskiewicz et al. 2019). Histo-blood group antigens represent another group of common terminal glycan structures, and *Helicobacter pylori* and rotaviruses/noroviruses use them for gastric and intestinal colonization in susceptible individuals, respectively (Ilver et al. 1998; Le Pendu and Ruvoën-Clouet 2020; Ramani et al. 2016, Rimkutė et al. 2020). *O*-linked glycans are natural carriers of such terminal structures because of the abundance of mucins in the digestive tract. Examples of pathogens that exclusively engage *O*-linked glycans are limited. Those that do most commonly recognize, degrade, and potentially feed on intestinal mucins. Pathogenic *E. coli* use glycopeptidase StcE to release intestinal mucins, allowing the bacteria to breach the mucin barrier and adhere to intestinal epithelial cells (Yu et al. 2012). Similarly, some species of mucin-degrading commensal bacteria, such as *Akkermansia muciniphila* and *Porphyromonas gingivalis*, encode glycopeptidases that recognize *O*-glycosylated peptides and cleave next to the glycan structure (Trastoy et al. 2020).

O-glycans on pathogens may also play a few defined roles in host–pathogen interactions. For example, enveloped viruses use the glycosylation machinery of the host cell for decoration of viral surface proteins, covering themselves in tolerogenic host-like glycans. A number of viruses, most prominently herpesviruses and filoviruses, are reported to have densely *O*-glycosylated surface proteins, and elongated *O*-glycan structures are required for sustainable propagation of HSV-1 (Bagdonaite et al. 2015, 2016; Feldmann et al. 1994). Surface-exposed viral envelope *O*-glycans may engage distinct host receptors for entry or transmission. Two specific *O*-linked glycans on HSV-1 fusion effector gB interact with the PILRα receptor (Wang et al. 2009; Furukawa et al. 2017; Arii et al. 2010). On other viruses, such as Ebola virus, *O*-linked glycans enhance infectivity and transmission by engaging macrophage galactose lectin on immune cells (Takada et al. 2004; Fujihira et al. 2018). Specific *O*-linked glycans in the stem region of a Hendra virus surface protein are suggested to be important for conformational stability and function (Stone et al. 2016). In the context of host immunity, dense *O*-glycosylation can shield immunodominant epitopes on viral proteins, although core-1 type *O*-glycans in the example of HSV-2 induce chemokine-mediated immune activation (Iversen et al. 2016; Machiels et al. 2011). These examples highlight some evolutionary adaptations of human pathogens to recognize and use host glycans, which has contributed to their diversity while allowing them to exploit the host glycosylation machinery. Some precise molecular mechanisms concerning mucin-type *O*-linked glycans remain to be elucidated.

2.5.2 *O*-glycan Structures in Inflammation and Cancer

Aberrant *O*-glycans are a well-established feature of cancer cells, and cancer-associated *O*-glycans, such as Tn (GalNAc-α1-S/T), STn

(NeuNAc2,6GalNAc-α1-S/T), and T (Galβ1,3GalNAc-α1-S/T), represent prime targets in cancer diagnosis and therapy. As an example, Tn occurs in 20–60% of cancers of the colon, lung, bladder, cervix, ovary, stomach, breast, and prostate, whereas very little Tn is found in mesenchymal cancers, blood cancers, and tumors of neurological origin, and no expression is found in normal healthy adult tissue (Ju et al. 2011; Springer 1984). Short *O*-glycans, however, are also present in early embryonic tissues, so that the structures were originally referred to as "oncofetal antigens" (Springer 1984). Furthermore, the aberrant *O*-glycans, especially STn, are detectable in several chronic inflammatory conditions, such as gastritis (Barresi et al. 2001), ulcerative colitis, and Crohn's disease (Itzkowitz et al. 1995, 1996; Karlen et al. 1998), and on circulating IgA in patients with IgA nephropathy (Novak et al. 2001). Only a few studies have addressed the mechanism underlying the presence of aberrant *O*-glycans in inflammation, but Tn and STn are believed to be induced by a pro-inflammatory microenvironment, including through cross-talk with tumor-associated macrophages (Cascio et al. 2017; Kvorjak et al. 2020). Forced expression of aberrant *O*-glycans, either through targeting elongating enzymes or through the redirection of GalNAc-Ts to the ER, aggravates the invasive behavior in model systems. This observation has led to speculation that chronic inflammation promotes tumorigenesis in part through inducing alterations in *O*-glycans (Hofmann et al. 2015; Radhakrishnan et al. 2014; Bergstrom et al. 2016; Gao et al. 2016). It is unclear, however, if both Tn and STn are presented on the cellular surface in inflamed tissue, as has been observed in epithelial cancers. More likely, only STn is expressed on the surface of inflamed tissue, possibly through deacetylation of the *O*-acetylated sialic acid residues present in normal colon. In contrast, Tn is primarily detected as an intracellular structure because of accumulation of the large mucins known to be upregulated in inflammation (Kufe 2009).

The expression of Tn and STn correlates with metastatic potential and poor prognosis, which is also often correlated with overexpression of the GalNAc-T3, -T6, and -T14 isoforms of initiating enzymes (Lin et al. 2017; Sheta et al. 2017; Wang et al. 2014; Konno et al. 2002; Gill et al. 2013). A mechanistic understanding of Tn and STn expression remains elusive. One possibility is loss or downregulation of elongating enzymes and required chaperones, such as core-1 β3-galactosyltransferase (C1GALT1), its obligate chaperone COSMC, and the core-3 synthase (B3GNT6). This process could naturally be combined with upregulation of the ST6GalNAc-T1 responsible for the biosynthesis of STn. With a few exceptions, the large majority of cancers do not harbor disruptive mutations in individual glycosyltransferases (Radhakrishnan et al. 2014).

Mutations have been observed in COSMC in a few colon and pancreatic tumor samples, as well as leukemia and melanoma cell lines (Ju et al. 2008a, b). However, the fact that tumors often overexpress core-1 structures (Sun et al. 2018) and many tumors simultaneously express both short and elongated glycans makes single disruptive mutations less likely to be the mechanism behind the observed alterations in *O*-linked glycans. An alternative explanation comes from the demonstration of changed expression and activity of glycosyltransferases through transcriptional and epigenetic regulation (Radhakrishnan et al. 2014; Vojta et al. 2016). Indeed, the transcriptional loss of the core-3 synthase B3GnT6 (Iwai et al. 2005) and upregulation of the STn synthase ST6GalNAc-T1 (Marcos et al. 2004) has been known for many years. In a similar manner, altered expression without the total loss of core-1 synthase activity would allow the simultaneous presence of short and longer *O*-glycans in various areas of the tumor. This hypothesis has encountered challenges, and several other mechanisms have been suggested, including relocation of GalNAc-Ts and other glycosyltransferases to the ER by a Src-induced COPI-dependent mechanism (Gill et al. 2010). Another suggested mechanism is based on cancer-induced Golgi disorganization and retrograde transport from the Golgi to the ER through a dynamic association of non-muscle myosin IIA with the cytoplasmic tail of Golgi glycosyltrans-

ferases coordinated by Rab6a (Petrosyan 2015). Other very likely explanations include changed localization of glycosyltransferases within the Golgi apparatus, and cancer-related changes in pH, both of which influence the presence of altered *O*-glycans (Kellokumpu et al. 2002; Campbell et al. 2001a; Gill et al. 2010, 2013). Regardless of the mechanism, changes in *O*-glycosylation, either acting inside cells or on the cellular surface, are expected to have a profound influence on many important events in cancer development and progression, including signaling, stability and function of receptor proteins, and adhesion to the matrix and to other cells (Dabelsteen et al. 2020; Radhakrishnan et al. 2014; Gill et al. 2013). These effects could arise through either site-specific glycosylation or presentation of elongated glycans with functional terminal glycan structures.

The functionality and expression of GalNAc-Ts change during carcinogenesis. For some GalNAc-Ts, including *GALNT5*, *-T12*, *-T15*, *-T16*, and *-T17*, rare inactivating somatic and germline mutations have been found (Wood et al. 2007; Guda et al. 2009; Abuli et al. 2011; Clarke et al. 2012). For the most part, however, other mechanisms probably account for the cancer-associated changes. These mechanisms include changes in microRNAs, as seen for GalNAc-T7, and altered transcriptional control, as observed for GalNAc-T3 (Izumi et al. 2003; Keita et al. 2013). The changes in expression are suggested to regulate specific signaling pathways and adhesive events involved in cancer progression, but verifying such associations with confidence is often difficult, not the least because of the multiple protein substrates and interplay with other isoforms. A few examples are available and include the effect of GalNAc-T1 in regulating proliferation of hepatocellular carcinoma and bladder cancer stem cells via the epidermal growth factor receptor and Sonic hedgehog signaling, respectively, whereas GalNAc-T2 is thought to regulate insulin-like growth factor 1 dimerization and signaling in neuroblastoma (Ho et al. 2014; Huang et al. 2015; Li et al. 2016). GalNAc-T2 is also proposed to influence epidermal growth factor receptor phosphorylation and signaling in gastric, oral, and hepatocellular carcinomas, and to affect hepatocyte growth factor receptor signaling in gastric cancer (Hu et al. 2018; Lin et al. 2014, 2016; Wu et al. 2011).

GalNAc-T3 has been associated with poor outcome and invasion in ovarian cancer and maintenance of pancreatic cancer stem cells, whereas the closely related GalNAc-T6 may be implicated in the progression of lung and breast cancers by affecting MAPK and estrogen receptor signaling (Barkeer et al. 2018; Deng et al. 2018; Song et al. 2020; Wang et al. 2014). Expression of GalNAc-T6 also has been linked to a cancer-like growth pattern in a colon cancer model, in which abolishing GalNAc-T6 normalized cell–cell adhesion and cell differentiation patterns (Lavrsen et al. 2018). This result suggests that aberrant GalNAc-T6 expression is involved in at least the early stages of oncogenic transformation (Lavrsen et al. 2018). These findings are in accordance with reports suggesting that GalNAc-T6 is involved in inducing the epithelial–mesenchymal transition and cadherin switching (Freire-De-Lima et al. 2011; Park et al. 2011; Tarhan et al. 2016), and a report showing a correlation between GalNAc-T6 expression and early-stage cancer tissue (Liesche et al. 2016).

GalNAc-T14 has been implicated in lung-specific breast cancer metastasis via bone morphogenetic protein signaling (Song et al. 2016). Because of the contextual function of GalNAc-Ts, however, extrapolating across cancer types is difficult. For example, GalNAc-T7 is downregulated in liver cancer, and silencing GalNAc-T7 in hepatic cancer cells induces cancer-promoting events (Shan et al. 2013). In contrast, the opposite pattern is found in cervical cancer (Peng et al. 2012). Such results make drawing general conclusions a challenge, but given the tremendous differences in signaling networks among different cancers and even among individual cells within tumors, they are not surprising.

2.5.3 Elongated *O*-GalNAc Glycan Structures in Cancer Biology

O-glycan core elongation and branching have also been associated with tumor progression. Although core-2 *O*-glycans are differentially regulated, with upregulation in some cancers such as prostate, colon, lung, and bladder cancer, and downregulation in breast cancer (Hagisawa et al. 2005; Vavasseur et al. 1994; Lloyd et al. 1996; Yousefi et al. 1991), the core-3 and core-4 *O*-glycans are generally downregulated (Iwai et al. 2005; Vavasseur et al. 1994, 1995). Many of these studies have not considered the different cells present in the tumor, including infiltrating immune cells, but they nevertheless illustrate the tissue- and context-specific functions of the different branched *O*-glycans. In addition to changes in core structures, there are increases in the level of terminal Lewis structures and poly-LacNAc extensions on other types of glycans such as *N*-linked glycans and glycosphingolipids, and elongated core-2 *O*-glycan structures in some instances. The appearance of these terminal structures leads to the acquisition of invasive and metastatic potential (Hagisawa et al. 2005; Hatakeyama et al. 2010; Miyamoto et al. 2013; Suzuki et al. 2012; Machida et al. 2001; Shimodaira et al. 1997).

Below, we briefly note a few characteristics related to the presence of elongated glycan structures, including Lewis structures and sialic acid, even though it is unclear if such elongated structures are present on *O*-glycans, *N*-glycans, or glycosphingolipids. Elongated glycans often carry Lewis structures, which include the monofucosylated Le^a and Le^x, and the di-fucosylated Le^b and Le^y structures (Fig. 2.1). A cancer-associated upregulation of Le antigens has been detected in several tissues, where it has been associated with invasiveness and poor prognosis (Yan et al. 2010; Motzer et al. 1988; Myers et al. 1995; Westwood et al. 2009; Brockhausen 2006). A similar overexpression of the sialylated variants of Le antigens has been detected in cancers and associated with aggressiveness and metastasis (Amado et al. 1998; Nakamori et al. 1993; Nakayama et al. 1995; Takada et al. 1993; Ogawa et al. 1994a, b; Renkonen et al. 1997; Gomes et al. 2013). This metastatic potential of cancer cells expressing SLe^x, and SLe^a seems to be mediated through binding to E- and P-selectins on vascular endothelial cells (Hakomori 2002; Kannagi 1997; Kim and Varki 1997; Iwai et al. 1993). Excessive sialylation is another general feature of cancer cells, and the extent of sialylation is associated with their invasive properties (Dall'Olio et al. 2014). One of the advantages of oversialylation is enhanced electrostatic repulsion resulting from the negative charge of sialic acid, which disrupts cell–cell adhesions and allows cells bearing these sialylated oligosaccharides to detach from the tumor (Pinho et al. 2009). Apha2,6-sialyltransferase-I (ST6Gal-I) and ST3Gal-III and -IV are responsible for sialylated Lewis structures and other sialylated glycan structures residing on both *O*- and *N*-glycans, and glycosphingolipids. Elevated levels of these molecules contribute to tumor progression, metastatic potential, and therapeutic resistance in cancer (Lin et al. 2002; Park and Lee 2013; Lee et al. 2012; Swindall and Bellis 2011; Park et al. 2012; Perez-Garay et al. 2010, 2013). Removal of sialylation, on the other hand, seems to circumvent this effect, as expression of the sialidase NEU1 correlates inversely with metastatic ability (Miyagi et al. 1994; Sawada et al. 2002) and leads to decreased invasion and Erk1/2 signaling in colon cancer cells (Uemura et al. 2009). These effects suggest a therapeutic role for sialidases as well as inhibitors of sialylation, which have been targets of several drug design strategies (Kijima-Suda et al. 1986; Chen et al. 2011; Hsu et al. 2005; Chang et al. 2006; Chiang et al. 2010; Mare and Trinchera 2004).

An emerging theme is the importance of cancer-associated glycans as an immune evasion strategy. During cancer growth, natural selection will retain features that promote the survival of cancer cells. Therefore, it is not surprising that some glycan alterations observed in cancer relate to evading immune surveillance. Many *O*-glycan features could play a role, with aberrant GalNAc-*O*-glycans such as Tn and STn being the most obvious. Tn-modified proteins bind to MGL preferentially expressed on tolerogenic antigen-

presenting cells such as dendritic cells (Mathiesen et al. 2018; Beatson et al. 2015; Napoletano et al. 2007; Tsuiji et al. 2002). Thus, Tn binding to MGL likely promotes survival through the creation of an immune-suppressive environment. Likewise, sialylated structures may play a role through Siglec interactions, which promote the presence and activation of regulatory T cells that inhibit immune activation (Paulson et al. 2012). In contrast to the effect observed by shortening glycans, elongated *O*-glycans also prevent immune activation in select cases (Madsen et al. 2013a, b). For example, in bladder cancer, the presence of core-2 *O*-glycan structures is associated with tumor progression (Tsuboi et al. 2011). A possible mechanism is that polyLacNAc on core-2 *O*-glycans binds galectin-3 and protects cancers from natural killer (NK)-mediated cell death by shielding the interaction between the glycosylated tumor-associated ligand MICA and the NK-activating receptor NKG2D (Tsuboi et al. 2011).

2.6 Therapeutics Based on GalNAc-*O*-Glycans

Given the many potential functions of *O*-GalNAc glycans, surprisingly few direct medical and therapeutic applications use this type of glycan. One of the most prominent examples is the targeting of the *O*-linked glycans of PSGL-1. Approaches to interfering with PSGL-1/P-selectin interactions include targeted antibodies and recombinant immunoglobulins, and possibly in the future, also glycosulfopeptide mimetics. Most recently, the strategy has been applied with success in sickle cell crisis based on the block between PSGL-1 on activated platelets and leukocytes (Ataga et al. 2017). Also, a benefit of blocking PSGL-1 and P-selectin interactions has been suggested for other diseases, such as cancer and inflammatory conditions, including the metabolic syndrome (Smith 2021 and Patel 2017).

Cancer is another area in which disease-specific changes in *O*-linked glycans have attracted substantial interest. Indeed, many clinically used serological biomarkers for cancer are *O*-glycoproteins. These include CA125 (MUC16) (Zurawski et al. 1988), prostate-specific antigen (Gilgunn et al. 2013), carcinoembryonic antigen (Goldstein and Mitchell 2005), and CA15-3 (MUC1) (Kumpulainen et al. 2002). As an example, CA125 is detected in 80% of patients with ovarian cancer (Nustad et al. 1996), whereas CA15-3 (MUC1) is used to monitor the course of breast cancer (Uehara et al. 2008). MUC1 belongs to the mucin family and contains a large, heavily glycosylated variable number tandem repeat region. As described above, under normal conditions, this region is heavily *O*-glycosylated, which shields the protein backbone from immune recognition. However, the *O*-GalNAc glycosylation status of these glycoproteins is altered during carcinogenesis, as described above (Fukushima et al. 2010; Jankovic and Milutinovic 2008; Saeland et al. 2012), which may be exploited to increase their specificity as cancer biomarkers. Glyco-mucin profiling, based on a proximity ligation assay, has been used to detect Tn and STn glycoforms of MUC16 and MUC1 in serous ovarian carcinomas. The combination could distinguish borderline and malignant tumors from benign lesions (Ricardo et al. 2015), suggesting that specification of the glycosylation status of cancer-associated glycoproteins could improve existing diagnostic assays. In this context, *O*-glycoproteomic studies with the identification of *O*-glycan sites on proteins may reveal cancer-specific, combined *O*-glycan/protein structures highly suited for application in cancer diagnosis and therapy.

As an alternative to the detection of antigen in the sera of patients, circulating autoantibodies may also be beneficial for early detection of cancer (Chapman et al. 2007; Anderson and Labaer 2005). The wide range of truncated *O*-glycan expression in cancer makes them prime targets for immunotherapy. Several initial immunizations with various *O*-GalNAc-glycans, such as Tn, STn, and T *O*-glycans, conjugated to immunogenic proteins have induced glycan-specific IgG and IgM antibody responses in mice and in patients with cancer (Adluri et al. 1995; O'Boyle et al. 1992; Fung et al. 1990; Henningsson et al. 1987; Lo-Man et al. 2001; Vichier-Guerre et al.

2000, 2003; Maclean et al. 1993; Slovin et al. 2003; Springer et al. 1994; Singhal et al. 1991). Other vaccines with multiple clusterings of short *O*-glycans can induce even more potent immune responses in mice (Ragupathi et al. 1999; Geraci et al. 2008; Lo-Man et al. 2001; Vichier-Guerre et al. 2000; Vichier-Guerre et al. 2003), nonhuman primates (Lo-Man et al. 2004), and patients with cancer (Slovin et al. 2003, 2005), supposedly because of their resemblance to the tumor cell surface or by mediating increased uptake into dendritic cells (Beatson et al. 2015; Tsuiji et al. 2002; Iida et al. 1999; Mathiesen et al. 2018; Napoletano et al. 2007). The only vaccine that has made it to clinical phase III trials thus far is the STn-keyhole limpet hemocyanin-conjugated vaccine Theratope®. Unfortunately, it failed to induce clinical improvement. Possible explanations are inadequate patient screening before inclusion (Heimburg-Molinaro et al. 2011) or the monovalency of the glycan structures used in this vaccine strategy (Fung et al. 1990; Sabbatini et al. 2000; Springer et al. 1994).

Another promising strategy, similar to that described above for the diagnostic markers, is to target the combined glycopeptide epitopes consisting of shortened *O*-glycans in combination with peptide epitopes. The advantage of combined *O*-glycopeptide epitopes is the generation of higher-affinity antibodies and increased cancer-specificity. The monoclonal Tn-MUC1 antibody 5E5 stains breast and ovarian tumor tissue (Tarp et al. 2007; Sorensen et al. 2006; van Elssen et al. 2010), and other *O*-glycan/mucin-specific antibodies have been developed (Danielczyk et al. 2006; Hanisch et al. 1995). One of these is PankoMab™, targeting Tn-MUC1, which reacts with a wide range of human carcinomas and demonstrates anti-cancer activity in preclinical trials (Danielczyk et al. 2006; Jeschke et al. 2012; Fan et al. 2010; Dian et al. 2009; Heublein et al. 2015). PankoMab™ and 5E5 react with GalNAc residues in different positions on the MUC1 backbone. The immunogenicity of *O*-glycopeptides in humans has been confirmed by the identification of autoantibodies targeting Tn-MUC1 (Pedersen et al. 2011b) and induction of Tn-MUC1-specific antibodies in patients with cancer after immunization with Tn-MUC1 peptides (Wandall et al. 2010; Sabbatini et al. 2007). The ability of these glycopeptides to overcome immunological tolerance in cancer suggests a possible role in active immunotherapy, but perhaps an even more promising strategy targeting cancer associated glycans is the development of potent antibody-based therapeutic strategies, including BiTEs and chimeric antigen receptors inserted into cytotoxic T cells (CAR-Ts). CAR-Ts directed to glycopeptide epitopes in MUC1 have indeed shown promise in preclinical animal models and are currently being tested in clinical trials (Posey et al. 2016).

Finally, the ability to control complex glycosylation pathways is especially interesting for the optimization of recombinant protein therapeutics. Several examples of how glycans can modulate half-life and tissue targeting are available, but most are related to the modification of the *N*-glycosylation pathway, and no specific examples of recombinant therapeutics in which the *O*-glycosylation pathway has been optimized exist. An alternative example is the introduction of pegylation using enzymatic GalNAc glycosylation at specific sites in proteins expressed in *E. coli* to ensure a longer half-life, as shown for granulocyte colony stimulating factor (CSF) and interferon-α2b (Defrees et al. 2006). In several other cases, it is possible to imagine that the specific introduction of biologically active *O*-glycan sites could improve the functionality and circulation time of protein-based drugs. For example, FGF23 could be produced with an *O*-glycan introduced by GalNAc-T3 to protect against cleavage and ensure sufficient circulation time. The same might be possible with enhancement of the circulatory stability of natriuretic peptides in the treatment of heart failure (Hansen et al. 2019). Another option is to use *O*-glycans as an attachment point for functional groups to secure a targeted uptake, as in the context of vaccines (Mathiesen et al. 2018).

2.7 Conclusion

Mucin-type *O*-glycosylation is one of the most abundant post-translational modifications not restricted to mucins, as witnessed by multiple gly-

coproteomic studies. This type of *O*-glycosylation is unique because it is initiated by a family of 20 differentially expressed isoenzymes, giving room to the regulation of function, but also making it notoriously difficult to study even to this day.

Knockout animal studies, and genome-wide analyses in humans have been instrumental in understanding which GalNAc-Ts have regulatory roles in specific tissues and processes. This has been supplemented by mass-spectrometry-based identification of differentially regulated targets, which has further elucidated a few well-defined molecular roles of isolated *O*-glycans, such as protection from proteolytic cleavage and modulation of ligand-binding and receptor activity. Aside from functions of isolated sites, clustered glycans provide structural support and stability for mucin domains, and binding sites for both endogenous and pathogen-derived carbohydrate binding proteins. Such interactions not only regulate important developmental programs in tissues and immune responses but also help maintain epithelial homeostasis with commensal organisms or, on the contrary, in some instances act as a weak spot for pathogen attachment. In addition, changes of *O*-glycan structures have been identified in several diseases, most notably cancer and inflammation. For example, in cancer, the disease-specific changes can be exploited to target tumor cells selectively by antibodies coupled with various effector functions, such as toxins, T cell engagers, or chimeric antigen receptor T cells. Similarly, blocking the carbohydrate-mediated interactions by either antibodies or mimetics can be used to prevent the attachment and recruitment of immune cells to areas with active inflammation.

In conclusion, mass spectrometry and robust genetic engineering have arguably been the breakthrough technologies allowing to appreciate the extent and isoform-determined variability in the *O*-glycosylation of proteins in different contexts, providing heaps of leads to follow for many years to come. However, many unanswered questions remain, including the occupancy of specific sites and the prospect of dynamic temporal regulation. Further advancements in mass spectrometry, as well as emerging single-cell analytical techniques, combined with bioinformatic approaches, may offer the next breakthrough in a more profound understanding of *O*-glycan function. Furthermore, it is becoming evident that simplified tissue models can help decipher the molecular mechanisms involved.

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Compliance with Ethical Standards

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Glycosphingolipids

3

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Abstract

Glycosphingolipids are amphiphilic plasma membrane components formed by a glycan linked to a specific lipid moiety. In this chapter we report on these compounds, on their role played in our cells to maintain the correct cell biology.

In detail, we report on their structure, on their metabolic processes, on their interaction with proteins and from this, their property to modulate positively in health and negatively in disease, the cell signaling and cell biology.

Keywords

Cancer · Ceramide · Glycosphingolipids · Neurodegeneration · Neuroprotection · Sphingosine

3.1 Glycosphingolipids

The history of glycosphingolipids (GSL) dates back to the late nineteenth century by J.L.W. Thudichum (1884), who reported, for the first time, on the isolation of unknown compounds from human brain. These compounds were found to be cerebrosides, that is, monoglycosylceramides (Fig. 3.1), but the knowledge of their structure remained enigmatic for a long time, before being resolved. It soon became clear that glycosphingolipids are a very large family of amphiphilic compounds, which present complex structure of glycans and lipids, involved in the positive and negative regulation of the biology of our cells (Thudichum 1884; Sonnino et al. 2006).

3.2 General Structure of Natural Glycosphingolipids

The term "glycosphingolipid" refers to lipids containing at least one monosaccharide residue and a sphingoid (Fig. 3.2) or a ceramide (Fig. 3.3).

Ganglioside nomenclature is in accordance with IUPAC-IUB recommendations (Chester 1998).

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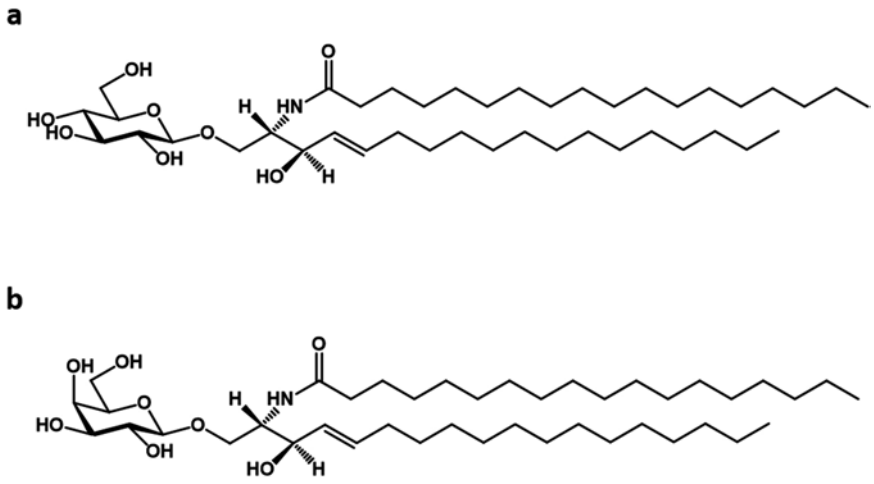

Fig. 3.1 Structure of glucosylceramide Glc-β-(1-1)-Cer (GlcCer) (**a**) and galactosylceramide Gal-β-(1-1)-Cer (GalCer) (**b**)

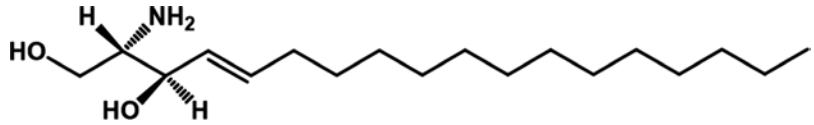

Fig. 3.2 Structure of sphingosine (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol,(Sph)

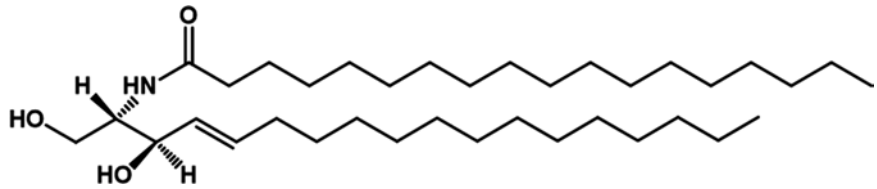

Fig. 3.3 Structure of ceramide (Cer)

Ceramides (Cer) are *N*-acylated sphingoids. The carbohydrate residue is attached to *O*-1 of the sphingoid by a glycosidic linkage. The trivial name of lyso-glycosylceramides is used for the glycosylsphingoid. The vast majority of glycosphingolipids are glycosylceramides but some glycosylsphingoids have been found in nature, which appear to be more abundant in patients with lysosomal storage diseases (Hikita et al. 2002).

Glycosphingolipids are classified as neutral or acidic. Among the latter, the main ones contain sialic acid (Fig. 3.4) (sialosylglycosphingolipids or gangliosides) or sulfuric acid (sulfoglycosphingolipids or sulfatides), but some contain uronic acid.

Sphingoids (Sph) are long-chain aliphatic amino alcohols, and the most common is the compound (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol. The abbreviation is *d*18:1 or C18-sphingosine. The corresponding saturated sphingoid (2*S*,3*R*)-2-aminoocta-1,3-diol originally called "dihydrosphingosine" is commonly

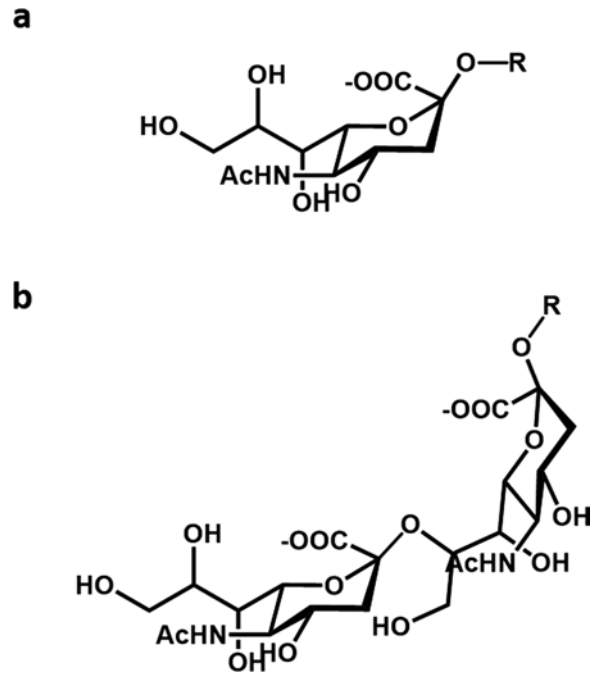

Fig. 3.4 Structure of: α -Neu5Ac (**a**) and α-Neu5Ac-(2-8)-α-Neu5A (**b**)

coded as sphinganine, C18-sphinganine or *d*18:0 (Charter et al. 1947). Small amount of sphingoids composed by different number of carbon atoms, with multiple double bonds, or hydroxyl, oxo, methyl substituents have been found in humans.

The main fatty acids of naturally occurring ceramides vary in chain length from C_{16} to C_{26} and may contain one or more double bonds and/or hydroxyl substituents at C-2 (Grösch et al. 2012; Wertz 2018). The neutral chain of glycosphingolipids on the basis of its structure defines the series of the glycosphingolipid to which additional sugars (e.g., sialic acid) or sulfate are connected. Table 3.1 shows the glycan series.

In all the glycans, sugars are defined with Roman numbering from I to V starting from the saccharide linked to ceramide, and the linkage position of an additional glycose residue to the sugar of the glycan series is coded with an apical Arabic number as reported in Table 3.2.

Sulfoglycosphingolipids are glycosphingolipids carrying a sulfate ester group, formerly called "sulfatides." The nomenclature of sulfatides is shown in Table 3.3. More detailed information on the nomenclature of glycosphingolipids established by the IUPAC-IUB are available in (Chester 1998).

3.3 Glycosphingolipid Metabolic Processes

Glycosphingolipids are widely distributed in all the cellular compartments, being membrane components of the subcellular fractions but are particularly abundant in the outer layer of the cell plasma membrane (PM). A small amount is also present in the cell cytosol in form of complexes with proteins (Sonnino et al. 1979; Merrill 2011).

De novo biosynthesis of glycosphingolipids first occurs in the endoplasmic reticulum (ER) where the highly hydrophobic ceramide is synthesized from serine and palmitoyl-CoA (in some tissues even stearoyl-CoA is the acceptor of ser-

Table 3.1 Glycosphingolipid series

Name and abbreviation	Structure
glucosylceramide	β-Glc-(1-1)-Cer
GlcCer	
Galactosylceramide	β-Gal-(1-1)-Cer
GalCer	
Lactosylceramide	β-Gal-(1-4)-β-Glc-(1-1)-Cer
LacCer	
gangliotriaosylceramide	β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gg_3Cer	
gangliotetraosylceramide	β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gg_4Cer	
gangliopentaosylceramide	β-GalNAc-(1-4)-β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gg_5Cer	
lactotriaosylceramide	β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Lc_3Cer	
lactotetraosylceramide	β-Gal-(1-3)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Lc_4Cer	
neolactotetraosylceramide	β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
nLc_4Cer	
neolactohexosylceramide	β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
nLc_6Cer	
globotriaosylceramide	α-Gal-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gb_3Cer	
isoglobotriaosylceramide	α-Gal-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
iGb_3Cer	
globotetraosylceramide	β-GalNAc-(1-3)-α-Gal-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gb_4Cer	
isoglobotetraosylceramide	β-GalNAc-(1-3)-α-Gal-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
iGb_4Cer	
globopentaosylceramide	β-Gal-(1-3)-β-GalNAc-(1-3)-α-Gal-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
Gb_5Cer	
Mollutetraosylceramide	β-GlcNAc-(1-2)-α-Man-(1-3)-β-Man-(1-4)-β-Glc-(1-1)-Cer
arthrotetraosylceramide	β-GalNAc-(1-4)-β-GlcNAc-(1-3)-β-Man-(1-4)-β-Glc-(1-1)-Cer

Table 3.2 Coding for some glycosphingolipids with additional glycose linked to the glycan series

Trivial abbreviation	Abbreviation
GM4	Neu5Ac-GalCer
GM3	II^3Neu5Ac-LacCer
GD3	$II^3(Neu5Ac)_2$-LacCer
O-acetyl-GD3	II^3[Neu5,9Ac_2-(2-8)-Neu5Ac]-LacCer
GM2	II^3Neu5Ac-Gg_3Cer
GD2	$II^3(Neu5Ac)_2$-Gg3Cer
GM1	II^3Neu5Ac-Gg_4Cer[a]
GM1b	IV^3Neu5Ac-Gg_4Cer
Fuc-GM1	IV^2αFucII^3Neu5Ac-Gg_4Cer
GalNAc-GM1	II^3Neu5Ac-Gg_5Cer
3′-LM1or LM1	IV^3Neu5Ac-nLc_4Cer
GD1a	IV^3Neu5AcII^3Neu5Ac-Gg_4Cer
GalNAc-GD1a	IV^3Neu5AcII^3Neu5Ac-Gg_5Cer
GD1b	$II^3(Neu5Ac)2$-Gg_4Cer
GD1b-lactone	II^3[Neu5Ac-(2-8,1-9)-Neu5Ac]-Gg_4Cer
Fuc-GD1b	IV^2αFuc$II^3(Neu5Ac)_2$-Gg_4Cer[b]
GT1a	$IV^3(Neu5Ac)_2II^3$Neu5Ac-Gg_4Cer
GT1b	IV^3Neu5Ac$II^3(Neu5Ac)_2$-Gg4Cer
O-Acetyl-GT1b	IV^3Neu5AcII^3[Neu5,9Ac_2-(2-8)-Neu5Ac]-Gg_4Cer
GT1c	$II^3(Neu5Ac)_3$-Gg4Cer
GP1c	$IV^3(Neu5Ac)_2II^3(Neu5Ac)_3$-$Gg_4Cer$[c]
GQ1b	$IV^3(Neu5Ac)_2II^3(Neu5Ac)_2$-$Gg_4Cer$
GQ1c	IV^3Neu5Ac$II^3(Neu5Ac)_3$-Gg_4Cer
AGM3	LacCer
AGM2	Gg_3Cer
AGM1	Gg_4Cer
GD1α	IV^3Neu5AcIII^6Neu5Ac-Gg_4Cer
GT1α	IV^3Neu5Ac$III^6(Neu5Ac)_2$-Gg_4Cer
Chol-1β	III^6Neu5Ac$II^3(Neu5Ac)_2$-Gg_4Cer
Chol-1α-a	IV^3Neu5AcIII^6Neu5AcII^3Neu5Ac-Gg_4Cer
Chol-1α-b	IV^3Neu5AcIII^6Neu5Ac$II^3(Neu5Ac)_2$-Gg_4Cer
GQ1α	$IV^3(Neu5Ac)_2III^6(Neu5Ac)_2$-$Gg_4Cer$
$FucGb_3Cer$	III^2αFuc-Gb_3Cer

[a]Neu5Ac, is always in α configuration
[b]$(Neu5Ac)_2$ = α-Neu5Ac-(2-8)-α-Neu5Ac
[c]$(Neu5Ac)_3$ = α-Neu5Ac-(2-8)-α-Neu5Ac-(2-8)-α-Neu5Ac

Table 3.3 Coding for sulfoglycosphingolipids

Trivial abbreviation	Abbreviation
SM4s	I^3sulfo-GalCer
SM4s-6	I^6sulfo-GalCer
SM4s-Glc	I^3sulfo-GlcCer
SM3	II^3sulfo-LacCer
SM2a	II^3sulfo-Gg_3Cer
SM2b	III^3sulfo-Gg_3Cer
SB1a	IV^3sulfoII^3sulfo-Gg_4Cer
$SMGb_4$	IV^3sulgo-Gb_4Cer
$SMGb_5$	V^3sulfo-Gb_5Cer
Sulfo-GM1	IV^3sulfoII^3Neu5Ac-Gg_4Cer
$SMUnLc_4Cer$	$IV^3$3sulfo-GlcU-Lc_4Cer
$SMUnLc_6Cer$	$VI^3$3sulfo-GlcU-Lc_6Cer

ine) and following in the Golgi compartment, where the hydrophilic oligosaccharide core is built up (Merrill 2011). The oligosaccharide chains are extremely variable displaying differences related to the number, structure and sequence of sugars, as well as the position of the sugar linkage and its configuration (Figs. 3.5 and 3.6). Figure 3.7 shows the general scheme for the biosynthesis of glycosphingolipids.

The biosynthesis of ceramide requires pyridoxal phosphate (vitamin B6), NADPH (vitamin B3), and oxygen (Stoffel 1970). The ceramide moves to the cytosolic layer of Golgi membrane transported by specific proteins (CERT) and by vesicle transport (Perry and Ridgway 2005). Here, the ceramide is first glycosylated into the cerebroside glucosylceramide (GlcCer) by GlcCer synthase or into the galactosylceramide (GalCer) by galactosylceramide synthase and the corresponding highly reactive UDP-sugar.

A portion of the GlcCer is directly transported to the PM inner layer by specific GlcCer cytosolic transferring proteins. At the PM, a specific flippase places GlcCer into the outer layer. Vesicular transport, flip flop, and/or four-phosphate adaptor protein 2 (FAPP2)-mediated transport allow most of GlcCer and GalCer to reach the lumen of the late-Golgi compartments where the synthesis of more complex glycosphingolipids takes place by sequential activity of glycosyltransferases specific for the acceptor and the nucleotide activated sugar (D'Angelo et al. 2007). Neosynthesized sphingolipids move via vesicular transport to the PMs, becoming components of the external leaflet (Capasso et al. 2017). Catabolism of sphingolipids occurs in lysosomes, from which simpler products, obtained in the degradation pathway, can escape.

Lysosomes are acidic membrane-provided intracellular organelles residing mainly in the perinuclear region. They contain about 60 different acid hydrolases, whose variety reflects the capability of the lysosomes to degrade multiple kinds of macromolecules, including nucleic acids, lipids, proteins, and glycosaminoglycans (Saftig and Klumperman 2009). Most of these enzymes are soluble, except for those involved in the lipid catabolism that are principally associated with the lysosomal membrane.

Lysosomal hydrolases are synthesized in the ER and then transported to the Golgi apparatus, where they are glycosylated and tagged with mannose-6-phosphate residues in the terminal position of the oligosaccharide chains (Fig. 3.8) (Braulke and Bonifacino 2009). Mannosylated enzymes are recognized by specific mannose-6-phosphate receptors (M6PRs) in the trans-Golgi network (Ghosh et al. 2003), and the M6PR–enzyme complexes are transferred to lysosomes through clathrin-coated vesicles. In the pre-lysosomal compartment, the increased acidity induces the release of enzymes from the M6PRs, which are recycled again in the Golgi apparatus. Another transport mechanism, mediated by Lysosomal Integral Membrane Protein 2 (LIMP-2), has been identified for the lysosomal enzyme β-glucocerebrosidase, responsible for the hydrolysis of the simplest glycosphingolipid glucosylceramide into glucose and ceramide (Reczek et al. 2007).

Lysosomes degrade intra- and extracellular macromolecules (Settembre et al. 2013) that reach these organelles by two main processes: autophagy and endocytosis. The main autophagic

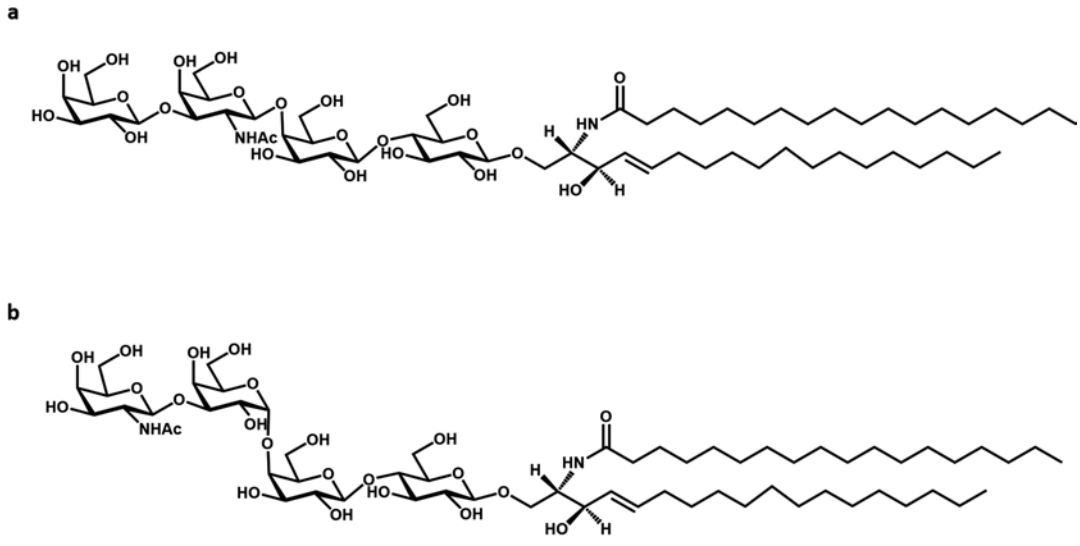

Fig. 3.5 Structure of: gangliotetraosylceramide, β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer, Gg4Cer (**a**) and globotetraosylceramide, β-GalNAc-(1-3)-α-Gal-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer, Gb_4Cer (**b**)

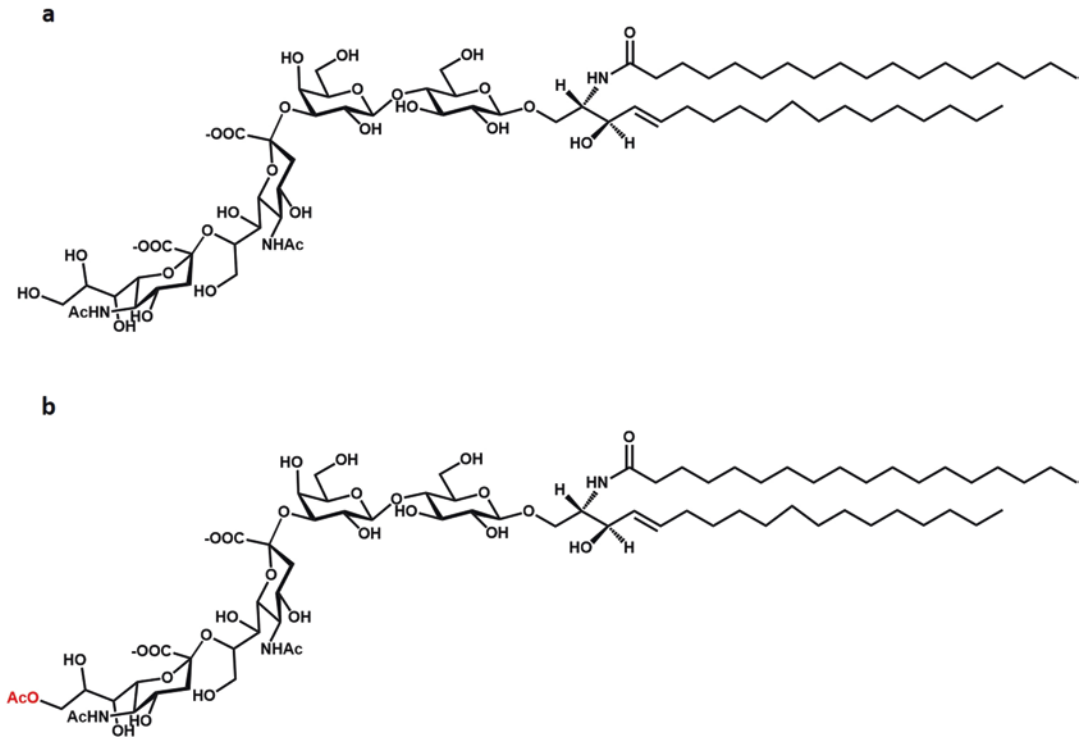

Fig. 3.6 Structure of GD3 α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer (**a**) and 9-*O*-Acetyl-GD3, α-Neu5,9Ac_2-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer (**b**)

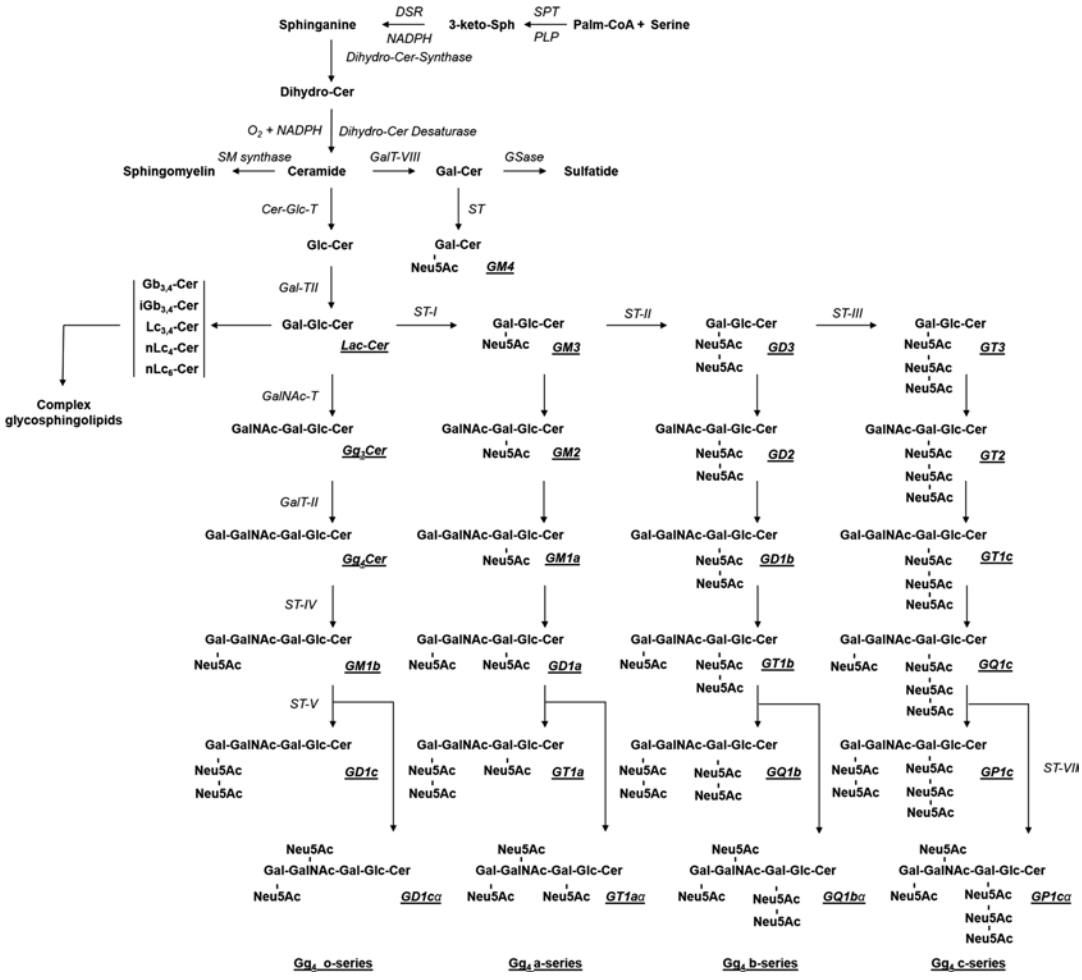

Fig. 3.7 Biosynthesis of glycosphingolipids: a general scheme

pathway is characterized by the formation of a double-membrane vesicle, called autophagosome, around the damaged organelle to be destroyed. Then, the fusion of the autophagosome with a lysosome allows the degradation of its content. On the other hand, endocytosis is the process involved in the internalization of extracellular material (Doherty and McMahon 2009). Endosomes undergo a maturation process characterized by multiple changes including exchange of membrane components, perinuclear localization, and decrease in luminal pH. After that, late endosomes fuse with lysosomes and lysosomal enzymes that degrade the extracellular material (Fig. 3.8).

Another important physiological role of endocytosis is its involvement in the plasma membrane turnover with particular regards for the transport of glycosphingolipids into the lysosomes for their degradation (Kolter and Sandhoff 2005). Within the lysosomes, glycosidases sequentially remove the glycosidic residues from the nonreducing end of the oligosaccharide chains.

For the proper glycosphingolipids catabolism, hydrolytic enzymes require the presence of activator proteins, a group of four small nonenzy-

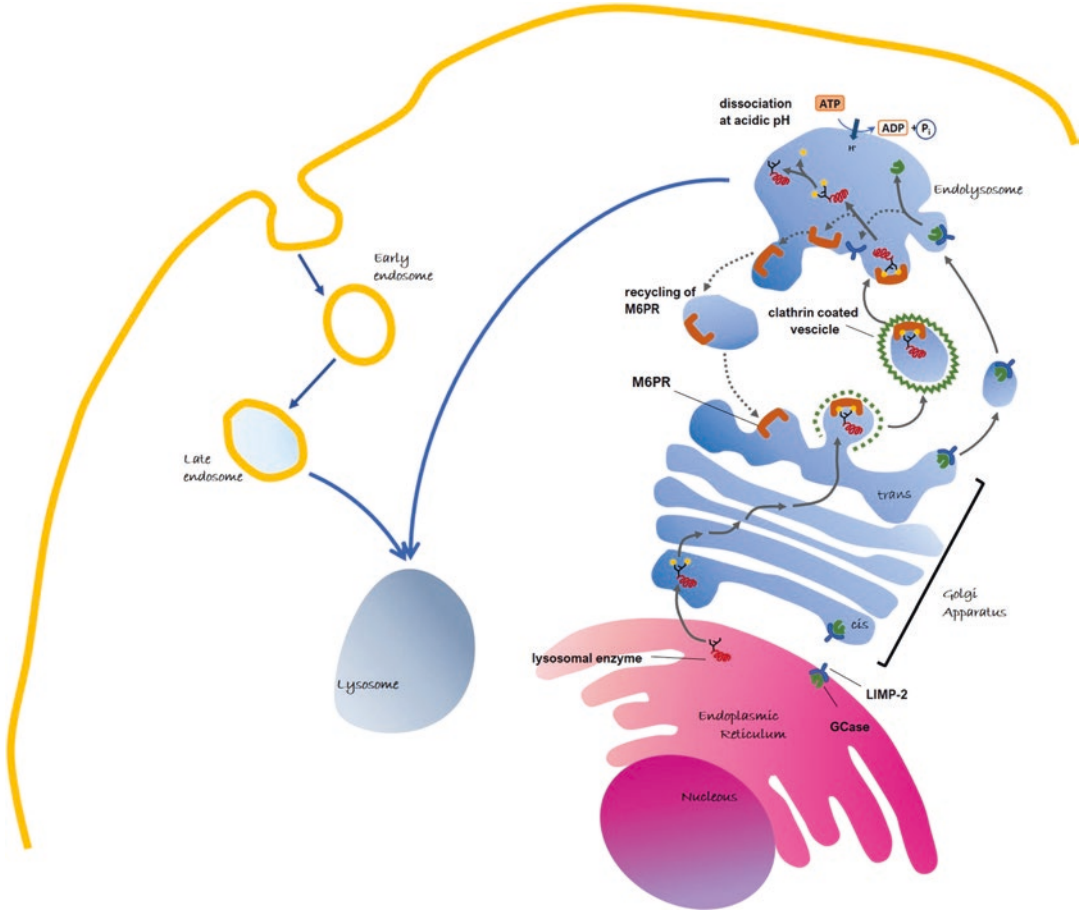

Fig. 3.8 Lysosome biogenesis and trafficking of hydrolytic enzymes. Lysosome biogenesis originates from the combination of biosynthetic and endocytic pathways as fusion between endolysosome and late endosome. Most of hydrolytic enzymes are synthesized in the endoplasmic reticulum and are glycosylated. In the Golgi apparatus they are tagged with mannose-6-phosphate (M6P) residues and, by M6P receptor (M6PR) mediated transport, can reach the endolysosomes in clathrin-coated vesicles. The acidity induces the release of enzymes from the M6PRs that is recycled to the Golgi apparatus. β-glucocerebrosidase is transported in a M6PR-independent way, by the interaction with the lysosomal integral membrane protein 2 (LIMP-2)

matic glycoproteins called sphingolipid activator proteins (SAPs) all deriving from the same precursor gene, plus the GM2 activator protein (Fig. 3.9) (Kishimoto et al. 1992). At the end of the degradative pathway of the oligosaccharide chain of glycosphingolipids, ceramide is hydrolyzed by acid ceramidase and saposin D to sphingosine and fatty acid (Ferlinz et al. 2001). The final products of glycosphingolipid degradation (sphingosine, fatty acids, and sugars) leave the lysosomes through specific membrane proteins and, in part, are recycled for the biosynthesis of new sphingolipids (Sonderfeld et al. 1985; Riboni et al. 1996; Tettamanti 2004; Kitatani et al. 2008; Maceyka et al. 2012).

The catabolism of glycosphingolipids depends on the recycling of the PM. Nevertheless, it is necessary to recall that only a minor portion of the endocytic vesicles becomes lysosomes, while the larger part rapidly reassociates with the PM. The glycosphingolipids' half-life is short in neurons, in fact for some gangliosides it is about 1 h, whereas in the case of fibroblasts it is around 2–3 days. In addition, it has been determined that

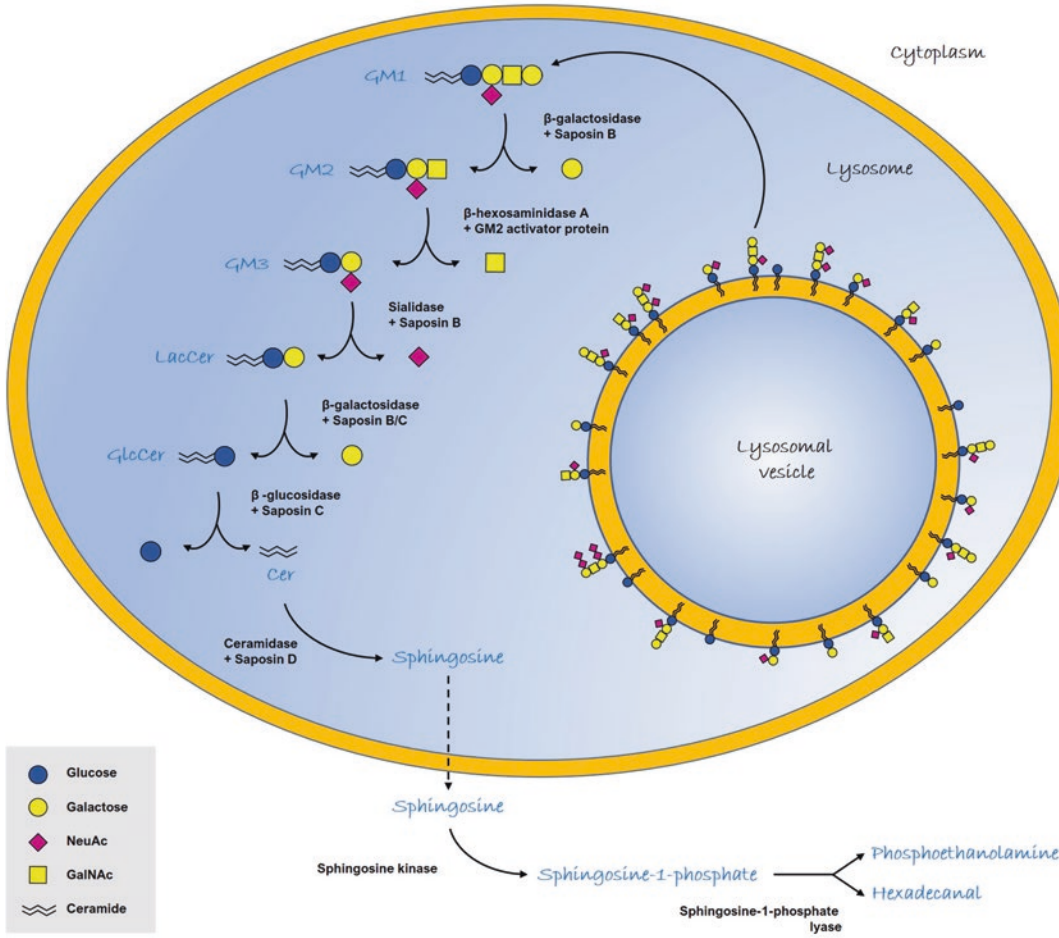

Fig. 3.9 Lysosomal glycosphingolipids' catabolism. The diagram reports the lysosomal catabolism of ganglioside GM1. Glycosidases sequentially remove the glycosidic residues from the non-reducing end of the oligosaccharide chains, thanks to the presence of activator proteins (Saposins – SAPs or GM2 activator protein) needed for the hydrolysis, whereas ceramide is hydrolyzed to sphingosine and fatty acid. In cytosol, sphingosine is phosphorylated and becomes substrate of a lyase, which converts sphingosine-1-phosphate in phosphoethanolamine and hexadecanal

up to 7–8% of the total cell sphingolipids are shed daily from cultured cells (Chigorno et al. 1997, 2006; Prinetti et al. 2000). Some of these are partially taken up by cells, becoming components of the membranes and contributing to modify their composition.

Accordingly, the regulation of PM sphingolipid content and pattern involves neosynthesis, catabolism, complex intracellular trafficking, and exchanges with the extracellular environment. Any change in these pathways contributes to the composition and organization of PM glycosphingolipids. In addition, both catabolic (hydrolases) and biosynthetic (transferases) glycosphingolipid enzymes have been found to be associated with the PMs, where they have been shown to exhibit activity on the membrane components.

A possible regulation of the glycosphingolipid biosynthesis at the transcriptional level through the control of the expression of glycosyltransferases or transporter proteins must be considered. This seems to be demonstrated by the parallelism

that exists in the changes in the expression of glycosyltransferases and the corresponding glycosphingolipid patterns that occur during neuronal development, oncogenic transformation, or the acquisition of drug resistance in cancer cells.

3.4 Glycosphingolipid–Receptor Interactions

As described above, glycosphingolipids are amphiphilic molecules mainly present in the PM with the hydrophobic ceramide inserted into the lipid bilayer and with the oligosaccharide portion protruding in the extracellular environment. Indeed, they not only are membrane structural components, but also play a fundamental role in signaling and regulatory pathways. Their chemical structure makes them ideal players in the role of mediators of information across the PM. The hydrophilic portion provides recognition sites for the interaction with other molecules into the lipid bilayer, while the hydrophobic portion, in its peculiar position, allows them to interact with the other components of the plasma membrane.

The unique lipid composition of a specific cell membrane could affect the function of a protein in two independent and distinct ways, either by modifying the properties of the membrane to achieve the physical parameters required for proper protein function or by specific protein–lipid interactions (Coskun and Simons 2011). These interactions may lead to different outcomes: (1) allosteric regulation of the protein conformation; (2) regulation of protein multimerization; (3) protein segregation to membrane domains; and (4) clustering of signaling molecules in proximity to their effectors (Russo et al. 2016).

For many years, it has been hypothesized that glycosphingolipids can modulate cell signaling pathways by interacting with hormones, membrane enzymes, intracellular transducers as well as transmembrane receptors, thus modulating their properties. With the advancement of methodological technologies, some of these interactions and the participation of glycosphingolipids to the cell signaling have been studied in detail and explained. Below are some of the best known and well characterized interactions between glycosphingolipids and receptor proteins associated with the cell PM. Particularly, the role of sialic acid containing glycosphingolipids, that is, gangliosides, in regulating the trophic factor-stimulated dimerization, autophosphorylation, and subsequent signal transduction of several tyrosine-kinase receptors is discussed.

3.4.1 Ganglioside GM1 and Trk Receptor

Neurotrophins and their receptors have been shown to be ganglioside GM1 interactors, contributing to explain the neurotrophic and protective effects of GM1, thanks to a specific protein–oligosaccharide interaction in the extracellular space (Ferrari et al. 1995; Mutoh et al. 1995; Farooqui et al. 1997; Bachis et al. 2002; Chiricozzi et al. 2017). Specifically, GM1 ganglioside ($II^3Neu5Ac\text{-}Gg_4Cer$, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-Cer) was shown to be necessary for the function of the tyrosine kinase receptor TrkA, specific for the nerve growth factor (NGF) neurotrophin. Earlier studies demonstrated that GM1 was able to activate TrkA receptor in different cell lines (Rabin and Mocchetti 1995; Mutoh et al. 1995) and primary neurons (Da Silva et al. 2005), suggesting that GM1 may mimic NGF activity in inducing neuronal differentiation, neuritogenesis, and neuroprotection (Sokolova et al. 2014). Importantly, using PC12 cells, with a very low GM1 content, it has been shown that the exogenous administration of GM1 strongly enhances NGF-mediated TrkA activation (Mutoh et al. 1995; Farooqui et al. 1997). Moreover, in cells lacking endogenous GM1, it has been demonstrated that NGF did not induce the autophosphorylation of TrkA, but the rescue of GM1 content recovered the responsiveness of Trk to its ligand (Mutoh et al. 2002). This evidence strongly suggests that GM1 is necessary for the normal functioning of Trk protein.

Colocalization of GM1 and Trk receptors in lipid rafts has been proposed to be necessary for TrkA phosphorylation in cultured cells (Mutoh et al. 1995), brain tissues (Duchemin et al. 2008), and in vivo (Duchemin et al. 2002; Mo et al. 2005). However, it has been recently shown that in neuroblastoma cells Neuro2a (N2a), TrkA does not belong to lipid rafts together with GM1, but its interaction with GM1 involves only the GM1 oligosaccharide chain and the extracellular portion of TrkA, which may flop down on the PM approaching the GM1 oligosaccharide chain (Chiricozzi et al. 2019a). Accordingly, 30 years ago, Schengrund observed that the GM1 oligosaccharide chain alone was able to induce neuritogenesis process in a neuroblastoma cell line (Schengrund and Prouty 1988). Following, Fantini and Yahi pointed out the presence of a GM1-binding domain in the extracellular domain of Trk, suggesting that GM1 could act as an endogenous activator of Trk receptors (Fantini and Yahi 2015). Specifically, Chiricozzi and coworkers recently reported that the specific sugar code of GM1-oligosaccharide (II^3Neu5Ac-Gg_4, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc, OligoGM1) acts as a bridge between the NGF and the TrkA receptor, directly participating in and stabilizing the interaction that leads to TrkA phosphorylation and to the activation of MAPK signaling (Fig. 3.10). This event leads to the activation of complex biochemical pathways that finally bring to neuronal differentiation and protection both in neuroblastoma cells and primary neurons (Chiricozzi et al. 2019a, b; Di Biase et al. 2020a; Fazzari et al. 2020).

Furthermore, GM1 also associates with TrkB, the tyrosine kinase receptor for brain-derived neurotrophic factor, BDNF (Pitto et al. 1998; Bachis et al. 2002) and with the GDNF receptor complex comprised of Ret, the tyrosine kinase component, and GFRα, a GPI-anchored coreceptor. The important role of GM1 in interaction with neurotrophic receptors is highlighted once again since the Ret association with GFRα was severely impaired in neurons totally or even partially devoid of GM1 (Hadaczek et al. 2015).

3.4.2 GM3 Ganglioside and EGF Receptor (EGFR)

Specific carbohydrate–carbohydrate and carbohydrate–protein interactions are known to be involved in the molecular mechanism mediating the inhibitory effect of GM3 ganglioside on the epidermal growth factor receptor (EGFR). Ganglioside GM3 ([α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc) has been reported to inhibit EGF-dependent cell proliferation in a variety of cell lines. Both in vitro and in vivo, this glycosphingolipid blocks the kinase activity of the EGF receptor (Miljan and Bremer 2002; Miljan et al. 2002). The ganglioside interaction site seems to be distinct from the receptor EGF-binding site, so this effect is not due to inhibition of binding of EGF to its receptor. It has been shown that GM3 interferes with the EGFR dimerization through a direct interaction (Yednak and Bremer 1994). It was reported that EGFR forms two different specific lateral interactions with GM3 at the extracellular plasma membrane level. One is a protein–carbohydrate interaction involving the terminal *N*-acetylneuraminic acid of the GM3 and the lysine 642 of EGFR localized in proximity to its transmembrane domain. Through this interaction, GM3 maintains the EGFR in its inactive state preventing receptor dimerization and activation without affecting ligand binding (Coskun et al. 2011). Second, GM3 interacts with the EGFR also by a carbohydrate–carbohydrate interaction involving the sialylated galactose of GM3 and the multi terminal *N*-acetylglucosamine residues on EGFR *N*-glycans. This interaction, although weaker, was demonstrated to inhibit EGFR autophosphorylation and activation (Fig. 3.11) (Kawashima et al. 2009; Russo et al. 2016).

3.4.3 Ganglioside GM3 and Insulin Receptor (IR)

Of clinical relevance is also the protein–carbohydrate interaction of ganglioside GM3 with the insulin receptor (IR) in adipocytes in a state of inflammation-induced insulin resistance (Nojiri

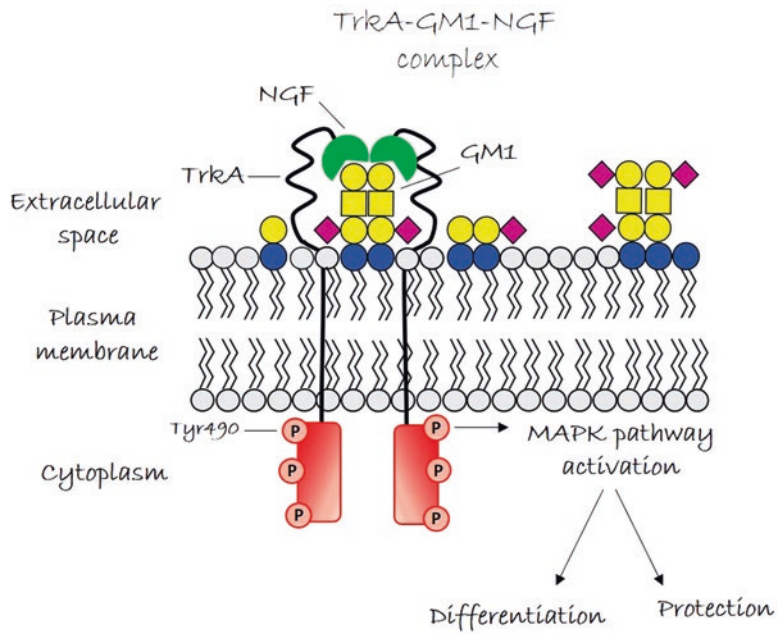

Fig. 3.10 Diagram of the GM1-TrkA mediated functions. GM1 ganglioside through its oligosaccharide chain stabilizes the TrkA-NGF complex on the cell surface triggering the phosphorylation of TrkA on tyrosine 490 (Tyr490) promoting MAPK signaling. This induces the activation of multiple intracellular pathways that finally lead to neuronal differentiation, protection and restoration. (Modified from Chiricozzi et al. 2017, 2019b). Glycosphingolipid sugar code is according to Varki et al. (2015)

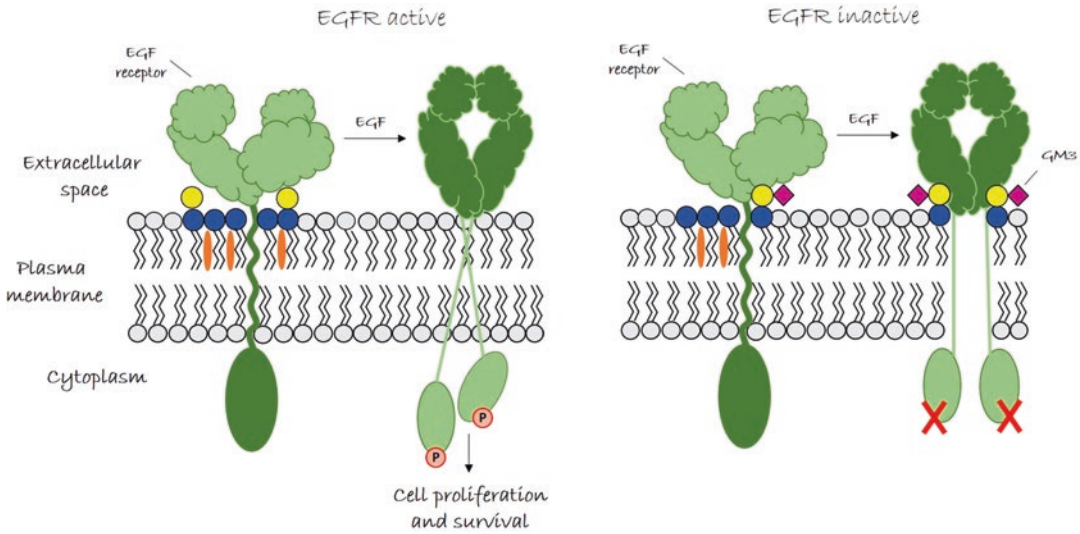

Fig. 3.11 Diagram of EGFR inhibition mechanism by GM3 ganglioside. On the left: EGF receptor association with sphingolipid cholesterol domains prevents the aberrant activation of EGFR signaling. EGF ligand binding promotes EGFR dimerization leading to the formation of an active dimer. On the right: When GM3 is present in the bilayer, the direct association of GM3 with the EGFR domain leads to the inactivation of the EGFR kinase activity. (Modified from Coskun and Simons 2011). Glycosphingolipid sugar code is according to Varki et al. (2015)

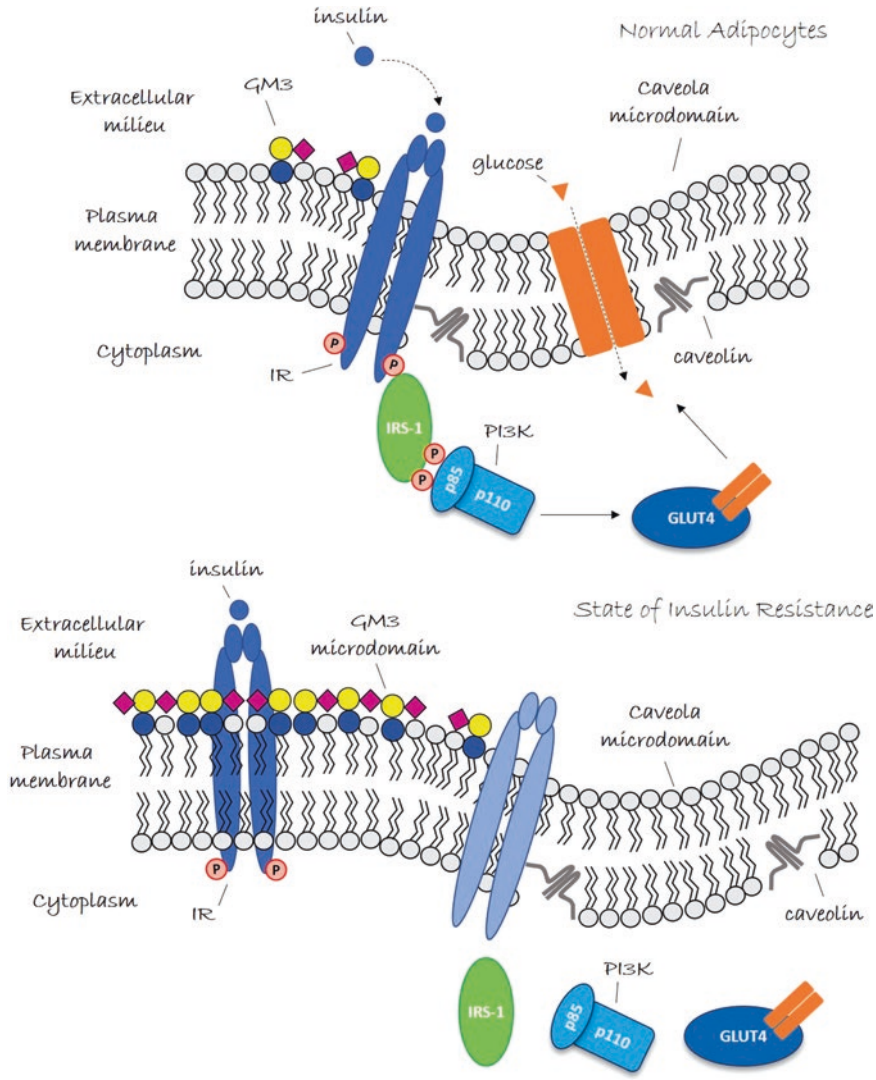

Fig. 3.12 Diagram of proposed mechanism behind the shift of Insulin receptor (IR) from the caveolae to the GM3 lipid rafts in adipocytes during a state of insulin resistance. Top: IR may be constitutively resident in caveolae via its binding to the scaffolding domain of Cav1 through the caveolin binding domain in its cytoplasmic region. Binding of IR and Cav1 is necessary for successful insulin metabolic signaling. Bottom: in adipocytes the localization of IR in the caveolae is interrupted by elevated levels of the endogenous ganglioside GM3 during a state of induced insulin resistance. (Modified from Kabayama et al. 2007). Glycosphingolipid sugar code is according to Varki et al. (2015)

et al. 1991; Tagami et al. 2002). An inhibitory effect of GM3 on insulin signaling has been reported both in cell systems (Tagami et al. 2002) and in mice lacking GM3 synthase that exhibit enhanced insulin signaling and are unaffected by insulin resistance induced by a high-fat diet (Yamashita et al. 2003).

Specifically, IRs are highly concentrated in caveolae microdomains where their binding to Cav1 is necessary for successful insulin signaling in adipocytes (Fig. 3.12) (Cohen et al. 2003). In adipocytes, in a state of TNFα-induced insulin resistance, there is an accumulation of ganglioside GM3 that is responsible for the elimination

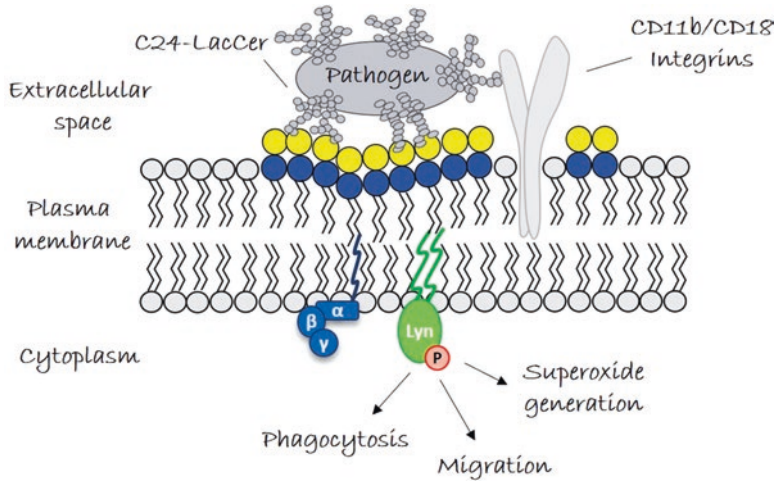

Fig. 3.13 Diagram of the LacCer-lipid raft mediated function in inflammatory response. Lactosylceramide (LacCer) with long fatty acid chain (C:24) presents into the lipid rafts of plasma membranes of neutrophil cells directly associates with the cytoplasmic protein Lyn via myristic/palmitic chains promoting its phosphorylation and activation of other proteins (Gi family protein). This specific C:24-LacCer/Lyn interaction finally leads to phagocytosis, superoxide generation and cell migration following CD11b/CD18 integrin translocation in C:24-LacCer microdomain in response to bacterial infection. (Modified from Chiricozzi et al. 2018)

of IR from the caveolae microdomains and the inhibition of insulin metabolic signaling (Tagami et al. 2002). The dissociation of the IR–Cav1 complex is caused by the interaction between the lysine 944 residue of IR, located just above the transmembrane domain, and the increased GM3 clustered at the cell surface (Kabayama et al. 2007).

3.4.4 Lactosylceramide, Lyn, and CD11b/CD18 Integrins

Neutral glycosphingolipids expressed on PM have been considered by many studies as the cell antenna for the recognition of pathogenic microorganisms (i.e., bacteria, fungi, and viruses). This interaction occurring at the cell surface represents the starting point for the cell signal response. Neutral glycosphingolipids can modulate the reactivity to stimuli both by acting as a membrane receptor for an extracellular ligand and by interacting with the other components of the cell PM.

Lactosylceramide [LacCer; β-Gal-(1-4)-β-Glc-(1-1)-Cer] is the most abundant neutral glycosphingolipid and is highly expressed on PM of human mature neutrophils (Brackman et al. 1995; Kniep and Skubitz 1998; Spychalska et al. 2003). It has been reported to act as a pattern recognition receptor (PRR) able to recognize pathogen-associated molecular patterns (PAMPs), leading to the activation of phagocytes' functions such as chemotaxis, phagocytosis, and superoxide generation (Iwabuchi et al. 2008; Yoshizaki et al. 2008; Nakayama et al. 2013). In particular, LacCer containing very long fatty acid chain of ceramide, C24:0 and C24:1, is the major molecular species of LacCer in human neutrophils (Iwabuchi et al. 2008). Specifically, C:24-LacCer has been demonstrated to form plasma membrane microdomains where specifically and directly interacts with Lyn protein, which is associated with the cytoplasmic layer via myristic/palmitic chains, whose activation finally leads to phagocytosis, superoxide formations, and migration mechanisms as response to pathogen infection (Fig. 3.13) (Iwabuchi and Nagaoka 2002; Iwabuchi et al. 2008; Chiricozzi et al. 2015). The long fatty acid of LacCer has been proposed to interdigitate with the acyl chains of the cytosolic

layer, reducing membrane thickness. The starting process required for LacCer-immunological function is the binding of pathogen ligand with the carbohydrate moiety that triggers transmembrane signaling. Consequently, a change in the thermodynamic properties of the distal carbons of the long fatty acid acyl chain allows direct interaction with the Src kinase Lyn on the cytosolic membrane surface. This is the first evidence of a direct interaction between a glycosphingolipid resident in the outer layer of the PM with a cytosolic-associated protein.

Importantly C:24-LacCer enriched PM microdomains are also fundamental for the CD11b/CD18 integrin mediated phagocytosis of nonopsonized microorganisms by human neutrophils (Iwabuchi and Nagaoka 2002; Nakayama et al. 2008). Upon binding of PAMPs expressed on pathogens, CD11b/CD18 is activated and undergoes a conformational change, resulting in the rearrangement of cytoskeletal proteins. The CD11b/CD18 subsequently translocates into LacCer-enriched membrane microdomains, allowing CD11b/CD18 to transmit stimulatory signals to Lyn through the interaction of LacCer with residues 514–553 in the C-terminal portion of the conserved domain of CD18 (Lu et al. 2001; Nakayama et al. 2008). These signaling cascades lead to the formation of actin-enriched phagocytic cups, resulting in phagosome formation (Nakayama et al. 2013).

3.4.5 Gb3 Globotriaosylceramide and Fas (CD95) Receptor

Another important neutral glycosphingolipid involved in the regulation of cell signaling response across the PM is globotriaosylceramide [Gb3, α-Gal(1-4)-β-Gal(1-4)-β-Glc-Cer] through its interaction with Fas receptor. Fas (CD95/APO-1/TNFRSF6) is a TNF receptor superfamily member, known as a key apoptosis activator, upon binding with its specific ligand (FasL). An efficient Fas activation of the apoptotic mechanism is subsequent to its internalization. Specifically, this process requires the interaction between Fas and a specific glycosphingolipid-rich membrane Gb3 raft. In particular, it has been demonstrated that Fas receptor contains a conserved extracellular glycosphingolipid-binding motif that interacts specifically with Gb3 regulating its internalization route and consequently the signals transmitted upon ligand binding (Lee et al. 2006; Chakrabandhu et al. 2008). When interacting with Gb3, Fas bounded to its ligand is internalized by clathrin-dependent endocytosis, which allows the transduction of cell death signal deriving from caspase-8 cascade activation (Kischkel et al. 1995; Mayor and Pagano 2007). In the absence of the lipid–receptor interaction, ligand-bound Fas is internalized by ezrin-mediated endocytosis, activating a progrowth signaling through MAPK cascade pathway (Chakrabandhu et al. 2008).

3.5 Aging: Glycosphingolipids in Span Life

Aging is defined as the progressive decline of the body's physiological functions, not necessarily linked to the chronological age (Gladyshev 2016). This is accompanied by an ever-decreasing ability of the organism to respond adequately to stimuli and thus the increasing of mortality rate (Flatt 2012). Aging is the first risk factor for a series of pathologies such as neurodegenerative disorders, cardiovascular and immune system diseases, and cancer (Ferrucci et al. 2020). Many factors are thought to contribute to mammalian aging including changes in gene expression, mitochondrial dysfunction, oxidative stress, shortening of telomeres, and accumulation of advanced glycation end-products. These processes are accompanied by substantial molecular and cellular changes that also affect glycosphingolipids. Changes in the content and composition of glycosphingolipids during aging have been mainly studied in few tissues, both in humans and in experimental in vivo and in vitro models. Here, we will make an overview on the variations that glycosphingolipids undergo during aging in different tissues.

3.5.1 Glycosphingolipids in Brain

Sialic acid-containing glycosphingolipids are particularly enriched in the nervous system playing both a structural and an essential functional role for the adequate development and maintenance of the nervous system (Schengrund 2015). The ganglioside content in the brain increases as development proceeds and simultaneously the expression pattern of gangliosides shifts from simple gangliosides, such as GM3 and GD3, which are mainly expressed in embryos and along the early post-natal life, to complex gangliosides, with GM1, GD1a, GD1b, and GT1b becoming the most represented ganglioside species in the adults (Ngamukote et al. 2007). In humans the content of gangliosides remains barely constant from 20 to 70 years, after when undergoes a progressive decline (Svennerholm 1994). Ganglioside changes in the brain are region-specific (Kracun et al. 1991; Svennerholm and Gottfries 1994). In fact, the frontal and temporal cortices and respective white matters show a reduction mostly of GD1a and GM1, and an increase of GM3 and GD1b within the cortices and of GD3 in the white matters. Additionally, a moderate GD1a and GM1 decrease in aged human hippocampus was detected (Kracun et al. 1991). Noteworthy, GD1a and GM1 deficiency has been reported to predispose to Parkinsonism (Wu et al. 2012; Ledeen and Wu 2018b). On the other hand, the cerebellar cortex showed a decrease in b-series gangliosides (GT1b and GD1b) during human aging (Kracun et al. 1991).

Moreover, imaging mass spectrometry studies highlighted the increase in the C:20 to C:18 ratio of fatty acid chain of ganglioside ceramide during brain aging (Sugiura et al. 2008), in accordance with previous founding in aged rat cerebellar granule neurons (Valsecchi et al. 1996). This, together with a progressive increase in the expression of the 3-keto-sphinganine synthase that uses stearoyl-CoA as substrate, leads to an increase in the C:20 to C:18 ratio of sphingosine. All this makes the brain gangliosides more hydrophobic and, consequently, the plasma membranes more rigid during aging. In rodents, the trend of brain ganglioside levels is similar but not identical to that of humans (Ohsawa 1989). In general, from 6 to 24 months of life, GD1a, GD1b, and GT1b remain constant and then drastically collapse up to 30 months. GM1, on the other hand, tends to increase up to 24 months and then only slightly decrease. Interestingly, GM1 tends to increase in the murine aged hippocampus and hippocampal synaptosomes (Yamamoto et al. 2008) such an increment supposedly to be associated with a seeding activity to the aggregation of misfolded proteins.

Considering ganglioside localization at the level of the presynaptic neuronal terminals modulating survival and neuronal transmission processes, the alteration of their content and composition associates with the reduction of neuronal function mainly through two mechanisms: trophic support insufficiency, since neurotrophic receptor activity is known to be modulated by gangliosides, and reduction of neuronal plasticity deriving from a limited vesicles capability to fuse with the membrane resulting in the lowering of synaptic vesicle release upon stimuli (Ledesma et al. 2012) This reflects in the cognitive decline that occurs with aging. Besides gangliosides, age-related increase in LacCer and GlcCer has been found in rodent brain together with a reduced acidic glucosylcerebrosidase activity (GCase, EC number 3.2.1.45) (Hallett et al. 2018). GCase is encoded by GBA gene and GBA haploinsufficiency is the highest genetic risk factor to develop Parkinson's disease (Sidransky and Lopez 2012), and interestingly, a significant reduction in GCase activity was found within the *substantia nigra* and putamen of elder people that could increase the vulnerability of dopamine neurons and lower the threshold for developing Parkinson's disease (Rocha et al. 2015).

3.5.2 Glycosphingolipids in Bone

Gangliosides were also described to be expressed in bone marrow mesenchymal stem cells (MSCs) and osteoblasts (Bergante et al. 2014, 2018). It has been documented that a-series ganglioside GD1a contributes to the regulation of osteoblast differentiation in MSCs (Kim et al. 2008). Series

b gangliosides (GD3, GD2, GD1b, andGT1b) were found in osteoclast precursors and primary murine osteoclasts, while they are not expressed in osteoblasts (Yo et al. 2019). Moreover, a down-regulation of GD3 synthase and b-series gangliosides is observed following induction of osteoclastogenesis mediated by receptor activator of nuclear factor kappa-B ligand (RANKL). Interestingly, the inhibition of GD3 synthase with the following ablation of b-series gangliosides appeared to prevent the age-related bone resorption in mice (Yo et al. 2019).

3.5.3 Glycosphingolipids in Kidney

Hernández-Corbacho and coworkers (Hernández-Corbacho et al. 2011) first described alterations in kidney sphingolipids in aged mice. Their data revealed that long-chain hexosilceramide (HexCer) and LacCer significantly increase during renal aging as well as in mouse aging brain and liver and in human fibroblasts obtained from elderly individuals. Even though the underlying mechanism associated with the age-related deterioration of kidney function and any other tissues is not known, some studies report HexCer and LacCer acting as mediators of inflammatory and apoptotic processes (Zhang and Kiechle 2004; Wennekes et al. 2009). Still scanty is the mechanism by which the alteration of glycosphingolipid metabolism occurs. Diet could play an in important role, as the caloric restriction was found to limit the accumulation of both renal HexCer and LacCer in aged mice (Hernández-Corbacho et al. 2011).

3.5.4 Glycosphingolipids in Endothelial Cells

Changes in glycosphingolipid composition of endothelial cells (EC) have been first reported by Sasaki and coworkers (Sasaki et al. 2015). Immunocytochemical and cytofluorimetric analysis revealed a GM1 increase in human cultured senescent EC. In addition, *B4GalNT1* gene (GM2-syntase, EC number 2.4.1.92) was upregulated. Furthermore, a decrease in the abundance of GD3 was observed in senescent ECs, suggesting that the GM2 and GM1 synthetic pathway is predominant in senescence. This is accompanied by impaired insulin signaling in senescent EC, with a slight reduction in the insulin receptor. Treatment of senescent EC with AMP-DNM, a glycosphingolipids' synthesis inhibitor, restored insulin signaling suggesting a possible negative impact of GM1 level alteration induced by aging. Even though this evidence remains to investigate more in detail the content of GM3 in the EC deriving from elderly subjects since GM3 is known to directly modulate the insulin receptor signaling (Kabayama et al. 2007).

3.5.5 Glycosphingolipids in Skin

The outer human epidermis layer, namely stratum corneum (SC), is enriched in lipids and is composed of 50% ceramides, 25% cholesterol, and 15% free fatty acids (Rogers et al. 1996). All three components are required for skin integrity, especially the ceramides, which play a crucial role in bilayer system formation (Feingold and Elias 2014). The SC ceramide derives from the metabolism mostly of GlcCer and sphingomyelin (SM) present in the innermost layers of the epidermis. These substrates are then metabolized by the action of glucosylceramidase and sphingomyelinase, respectively (Holleran et al. 2006). During aging, the skin becomes thinner and dry and decreases the ability to recover from lesions. An overall reduction in SC lipid content has been found in elder subjects as well as in aged mice, without any alteration in composition of each lipid species (Ghadially et al. 1995). A decrease activity of the enzymes involved in the synthesis and catabolism of GlcCer have been found in aged skin and pathological conditions suggesting a lower substrate availability for ceramide production (Holleran et al. 2006). Interestingly, the oral administration of cereal lipid extract enriched in GlcCer appeared to increase ceramide content in the SC of aged mice and to ameliorate recovery ability after injury (Bizot et al. 2017).

3.6 Glycosphingolipids in Disease

In the previous paragraphs, we reported the role played by glycosphingolipids in regulating signal transduction. Hence, here comes the importance of the correct glycosphingolipidic content and pattern of living cells and the correct glycosphingolipid topology. These three points depend on the balanced expression of a multitude of enzymes necessary for their biosynthesis, catabolism, and intracellular transport. Any change in the metabolic pathway could affect glycosphingolipids' content and composition resulting in their reduced availability on the cell surface and/or overexpression of some species. All these events predispose to impairment of cell functioning leading to disease onset.

3.6.1 Glycosphingolipids and Neurodegeneration

Considering the increase of population age, in the last decades, a huge number of studies have focused on neurodegeneration, a phenomenon consisting in the progressive loss of structure and function of neurons leading to the onset of neurodegenerative disorders.

Since sphingolipids are key cell signaling molecules enriched in neuronal membranes, their biology and alterations have been extensively studied in the context of neurodegeneration (Piccinini et al. 2010; Breiden and Sandhoff 2018; Magistretti et al. 2019). Indeed, different neurodegenerative disorders are characterized by central and peripheral alterations of the composition and metabolism of sphingolipids, particularly of glycosphingolipids such as glycosylceramide, sulfatides, and gangliosides (Wang and Bieberich 2018).

Altered levels of sphingolipids have been found both in acute and in chronic neurodegeneration.

In response to acute injuries (i.e., stroke, spinal cord injury, and traumatic brain injury), the activation of sphingomyelinases and glycosidases accompanied by increased levels of ceramide and glycolipids has been reported (Novgorodov and Gudz 2009; Horres and Hannun 2012; Gu et al. 2013; Brunkhorst et al. 2015; Ong et al. 2015; Jones and Ren 2016; Roux et al. 2016; Barbacci et al. 2017; Abe et al. 2018; Sajja et al. 2018). On the other hand, loss-of-function alterations of enzymes involved in the hydrolytic degradation of glycosphingolipids can lead finally to chronic neurodegeneration due to sphingolipid aberrant accumulation within lysosomes and related cellular compartments (Ariga et al. 2008; Mielke and Lyketsos 2010; Haughey 2010; Mencarelli and Martinez-Martinez 2013; Farfel-Becker et al. 2014; Schnaar 2016; Spassieva and Bieberich 2016; Sandhoff 2016; Arenz 2017; Di Pardo and Maglione 2018; Grassi et al. 2019) (see section "Glycosphingolipidosis").

Besides its association in sphingolipidosis-derived neurodegeneration, several evidence highlighted the central role of gangliosides both as therapeutic agents and as putative initiators of neurodegeneration via subnormal levels (Ledeen and Wu 2018a, b; Magistretti et al. 2019).

The involvement of a-series gangliosides, and in particular of GM1 and its catabolic precursor GD1a, represents a common denominator for at least three neurodegenerative disorders: Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (Ledeen and Wu 2018a, b; Magistretti et al. 2019).

3.6.1.1 Parkinson's Disease

PD is a disorder characterized by accumulation of α-synuclein (α-syn) fibrillary aggregates and progressive degeneration of nigrostriatal dopaminergic neurons that finally lead to motor and cognitive dysfunctions (Del Tredici et al. 2002; Ledeen and Wu 2018a, b; Zesiewicz 2019).

About 5 to 10% of PD cases have a known genetic origin, while the vast majority, defined as sporadic PD, has only age as the major risk factor (Stern et al. 2012; Ledeen and Wu 2018b). Up to now, the most common genetic rick factor of PD is represented by mutations in the *GBA* gene, coding for the lysosomal β-glucocerebrosidase enzyme (GCase), for which the 3–5% of PD patients carry heterozygous mutations.

Until 20 years ago, the *GBA* gene was considered responsible for only the Gaucher's disease (GD), one of the most common lysosomal storage disorders. GCase catalyzes the hydrolysis of GlcCer to ceramide and glucose mostly within lysosomes and partially at PM level. Homozygous or, more commonly, combined heterozygous mutations in *GBA* gene, generally, cause the enzyme to be defective leading to the accumulation of the substrate, responsible for the multiorgan clinical manifestations of the disease.

The initial discovery of Parkinsonism in a subset of adult onset type I GD patients suggested a possible pathogenic link between the two disorders (Neudorfer et al. 1996; Tayebi et al. 2003; Sidransky 2005). Neuropathological analysis of these patients revealed the presence of α-syn positive Lewy bodies (Wong et al. 2004), suggesting the involvement of αsyn aggregation. Importantly, several additional genetic studies in large patient cohorts demonstrated that patients with Parkinsonism have an increased incidence of *GBA* mutations (Lill et al. 2009).

Despite the efforts of researchers, to date the mechanism underlying the relation between *GBA* mutations and the development of PD is far to be completely understood and different hypotheses have been explored. Since GCase resides and acts within endolysosomal compartment, its incorrect folding could induce an impairment and engulfment of ER and lysosomes which in turn would trigger a stress response in the dopaminergic neurons leading to their damage and death (McNeill et al. 2014). In addition in GBA-PD and non-GBA-PD, GCase shows a significant reduction of its catalytic activity resulting in a loss of function that seems to be associated with a gain of a toxic–cytotoxic function resulting in an aberrant positive feedback loop involving GCase, alpha-synuclein (α-syn) and the nonmetabolized GlcCer (Mazzulli et al. 2011). The intracellular GlcCer levels control the formation of soluble toxic α-syn assemblies in cultured neurons and mouse and human brain, leading to neurodegeneration. The elevation and formation of α-syn assemblies further contributes to a pathogenic cycle by inhibiting the lysosomal maturation and activity of normal GCase, resulting in additional GlcCer accumulation and augmented α-syn oligomer formation. The central role of not correctly metabolized GlcCer accumulation in the α-syn pathology continues to emerge from recent reports according to which the accumulation of GlcCer even in the absence of a direct GBA mutation is sufficient to induce a conformational change of the physiological aggregates of α-syn in pathological oligomers, with a tendency to form fibrils. Even more important for the therapeutic perspectives, the reduction of GlcCer induces a reversion of the pathological conformers of α-syn into the physiological ones (Zunke et al. 2018). It follows that strategies to restore/maintain GCase activity and reduce GlcCer levels are currently under exploration to treat the misfolding and pathogenic aggregation of α-syn in PD (Riboldi and Di Fonzo 2019).

Although sporadic PD etiopathogenesis is complex and to date poorly understood, and both genetic and environmental factors have been reported to play a synergistic role, a new theory confers a crucial role to GM1 ganglioside. This ganglioside highly enriched in neurons exhibits a physiological progressive decline with aging and/or epigenetic influences (Svennerholm and Gottfries 1994). However, its decrease below critical thresholds may induce the deregulation of key molecular mechanisms leading to neuropathological dysfunction and finally to PD onset (Ledeen and Wu 2018a, b; Chiricozzi et al. 2020). Accordingly, PD patients reported a decreased expression of genes involved in GM1 synthesis, such as *B4galnt1, B3galnt4*, and *St3gal2*, accompanied by a reduction of GM1 in central and peripheral nervous tissues (Ledeen and Wu 2018b; Schneider 2018). Besides the clinical data, the consequence of GM1 insufficiency is illustrated in a newly presented mouse model of sporadic PD that was obtained from the heterozygous disruption of the *B4galnt1* gene causing a partial depletion of GM1 comparable to those found in PD patients (Wu et al. 2012; Hadaczek et al. 2015; Ledeen and Wu 2018a, b). This condition is sufficient to accurately recapitulate behavioral and biochemical PD phenotype that was found to be recovered by administration of

LIGA-20, a membrane-permeable analogue of GM1 (Wu et al. 2012; Ledeen and Wu 2018b).

The neurorestorative and neuroprotective potential of GM1 ganglioside was highlighted by different studies using widely accepted in vivo PD models, including mice and nonhuman primates exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a neurotoxin impairing mitochondrial function (Hadjiconstantinou et al. 1989; De Girolamo et al. 2001; Nicotra and Parvez 2002; Meredith and Rademacher 2011), and rats overexpressing human A53T mutant α-syn via adeno-associated viral vector (Schneider et al. 2019). At molecular level, several neuronal functions can become gradually compromised as GM1 levels recede (Ledeen and Wu 2018a, b; Chiricozzi et al. 2020). In PD context, a particular attention was given to the signaling mediated by neurotrophic receptors and the α-syn interaction. Indeed, it has been hypothesized that the reduced level of plasma membrane GM1 in PD neurons may trigger the neurodegenerative process due to a failure in trophic signaling together with a reduction of α-syn clearance (Bartels et al. 2014; Hadaczek et al. 2015). Accordingly, GDNF signaling, found to be impaired both in the *substantia nigra* from PD patients and *B4galnt1* PD mouse model, was rescued through the administration of the GM1 analogue LIGA20 (Hadaczek et al. 2015). In addition, studies have highlighted the specific inhibition of α-syn fibril formation mediated by GM1 binding and the key role of *N*-acetylation of human α-syn in enhancing GM1 binding and specificity (Martinez et al. 2007; Bartels et al. 2014).

However, although a direct correlation between GM1 and the loss of neurotrophic signal is quite evident both in vitro and in vivo (Schengrund and Prouty 1988; Schneider and DiStefano 1994; Wu et al. 2004, 2005, 2012; Schneider et al. 2015a), to date the molecular mechanisms underlying these GM1 effects remain to be elucidated. In this context, difference in vitro and in vivo evidence pointed out that the functional moiety responsible for activating the GM1 neurofunctions resides in its oligosaccharide chain (Schengrund and Prouty 1988; Schneider and DiStefano 1994; Wu et al. 2004, 2005, 2012; Hadaczek et al. 2015; Schneider et al. 2015b; Di Biase et al. 2020b; Fazzari et al. 2020). This evidence led to hypothesize that sporadic PD pathogenesis may be due to PM GM1 deficiency that causes an alteration of the molecular interaction between the GM1 oligosaccharide and specific PM proteins. Since GM1 is involved in the regulation of numerous neuronal pathways, the loss of specific GM1 oligosaccharide–protein interactions may gradually compromise several functions.

As reported above, recently a new theory emerged regarding a possible role for ganglioside GM1 and its precursor (i.e., ganglioside GD1a) in the etiopathogenesis of sporadic form of PD, due to a decrease of their expression under a specific threshold level during aging (Ledeen and Wu 2018a, b).

Accordingly, this reflects the partial success of the GM1 replacement therapy in clinic. Starting from the 90s, Schneider and coworkers carried out a trial with the aim to check the short- (24 weeks) and long-term (120 weeks) effects of GM1 treatment on PD: improve symptoms, delay disease progression, and partially restore damaged brain cells (ClinicalTrials.gov NCT00037830; (Schneider et al. 2010). Up to 100 patients were daily treated intravenously or subcutaneously with 100–200 mg of GM1 for up to 5 years (Schneider et al. 2010). The GM1 treatment brought positive effects, like a partial restoration of dopamine transporter functional level in the striatum, improvement of motor symptoms, and lowering the disease symptom progression (Schneider et al. 2010, 2013, 2015a). The study results provided indications that GM1 may improve motor symptoms and may also have potentially disease modifying effects on PD patients. However, since the small cohort of patients enrolled in these trials, the data obtained were not clear enough to authorize GM1 as an official drug for the treatment of PD.

Additionally, an important and fundamental problem in the peripheral administration of GM1 ganglioside is that for its chemophysical properties (i.e., amphiphilic characteristic) it hardly crosses the blood–brain barrier (BBB) and thus a

too small quantity of GM1 may reach the damaged neurons. It is plausible to think that the positive results obtained in GM1 trials on PD patients are mainly due to the altered-inflamed BBB areas, typically observed in patients with advanced pathology (Saulino and Schengrund 1994; Desai et al. 2007; Erdö et al. 2017).

In conclusion, although different studies and clinical data reported the therapeutic action of systemically administered GM1 (Schneider et al. 1992, 2015a; Herrero et al. 1993; Rothblat and Schneider 1998), it remains to be verified whether GM1 can reach brain regions by crossing intact BBB (Ghidoni et al. 1986; Svennerholm et al. 1990; Saulino and Schengrund 1994; Polo et al. 1994).

To facilitate the brain internalization different strategies can be developed, including the optimization of drug delivery and the design of modified-GM1 able to more efficiently cross BBB.

In 2012, Wu et al. demonstrated that the addition of a dichloroacetyl group linked to the sphingosine amino group instead of the acyl chain generates a membrane-permeable analogue of GM1, LIGA-20, able to maintain different GM1 neuroproperties (Wu et al. 2012). In addition, while the toxicity associated with long-term treatments hampered its use, the effectiveness of LIGA-20 suggested that the ceramide moiety is not critical for preserving the GM1 functions (Schneider and DiStefano 1994; Wu et al. 2004, 2005; Hadaczek et al. 2015). In well accordance, recent studies have highlighted that the oligosaccharide portion of ganglioside GM1 is able to replicate the neurotrophic and neuroprotective properties of the entire GM1 both in vitro and in vivo (Chiricozzi et al. 2017, 2019a, b, c; Di Biase et al. 2020a, b; Fazzari et al. 2020). In this scenario, the GM1 oligosaccharide maintains all the beneficial effects of the ganglioside from which it derives but loses its amphiphilicity acquiring the ability to effectively reach the central nervous system, thus demonstrating a strong therapeutic potential.

3.6.1.2 Alzheimer's Disease

AD is the most common neurodegenerative disorder characterized by impairment in memory and cognition due to the presence of abundant extracellular amyloid β plaques and neurofibrillary tangles triggering neuronal degeneration (Serrano-Pozo et al. 2011).

In this context, the analysis of samples from patients affected by dementia of Alzheimer type compared to controls led to identification of a significant reduction of all gangliosides in hippocampal, entorhinal, posterior cingulate, visual and prefrontal cortex, with a preferential decrease of b-series gangliosides (GD1b, GT1b) (Crino et al. 1989). On the other hand, the ganglioside composition in AD frontal cortex was found to be similar to controls, with minor exception for GQ1b and GT1b that resulted decreased (Brooksbank and McGovern 1989). By employing a peculiar method for ganglioside quantification (TLC–Blot and MALDI-TOF mass spectrometry), the depletion in b-series gangliosides was identified in AD hippocampal gray matter, while the level of the a-series gangliosides (GM1 and GD1a) was not affected (Valdes-Gonzalez et al. 2011).

The decrease of all gangliosides in the temporal and frontal cortex and nucleus basalis of Meynert (Kracun et al. 1991) or a mild decrease of a-series gangliosides in the temporal lobe of late-onset patients (Svennerholm and Gottfries 1994) were also reported. In addition, another study identified an increase of simpler gangliosides (i.e. GM2 and GM3) in AD cortex indicating, possibly, the acceleration of gangliosides lysosomal degradation and/or the presence of reactive astrogliosis typical of neuronal death process (Kracun et al. 1992). Data regarding the GM1 levels and function are controversial: it has been both negatively and positively linked to AD pathogenesis (Ariga et al. 2008). Different studies have reported the GM1 capability to bind amyloid β-protein and seed its fibrillogenesis to the aggregated form, the neuropathological hallmark of AD tissues (Hayashi et al. 2004; Ngamukote et al. 2007; Ariga et al. 2008). In addition, a cytotoxic effect was reported in mouse embryonic neural stem cell coexposed with GM1 and amyloid β (1–40) peptide (Yanagisawa et al. 2010). On the other hand, independent in vitro studies reported the protec-

tive action of GM1 ganglioside in the presence of amyloid β fibrillae (Ariga et al. 2008; Ledeen and Wu 2018b), indicating the GM1 capability to increase cell viability (Sokolova et al. 2007), to modulate inflammation by inhibiting amyloid β-induced release of proinflammatory cytokines (Ariga and Yu 1999), and to prevent toxicity triggered by fibrillar amyloid β via modulating the GSK3β-induced apoptotic pathway (Kreutz et al. 2011).

In addition, the crossing of homozygous GD3 synthase knockout mice, lacking the b- and c-series gangliosides, with a double-transgenic (APP/PSEN1) mouse model of AD has led to the elimination of amyloid β plaques and to a behavioral performance overlapped to wild-type mice (Bernardo et al. 2009). These results suggested not only that GD3 synthase could be a valuable therapeutic target to counteract AD but also that these improvements might be attributed to increase of GM1 and GD1a seen in the triple-transgenic mice (Bernardo et al. 2009).

Accordingly, GM1 administration to Wistar rat injected with amyloid β1-40 improved spatial learning and memory deficits and inhibited the oxidative stress and lipid peroxidation (Yang et al. 2013). Further in vivo studies suggested that the beneficial effects of GM1 against amyloid β-derived toxicity could reside in the enhanced of amyloid β clearance via autophagy (Dai et al. 2017) or the elimination by brain phagocyte microglia of amyloid β trapped in glycosphingolipid-rich exosomes (Yuyama et al. 2014).

Finally, the intraventricular injection of GM1 in five early-onset AD patients determined a significant amelioration of motor performance and neuropsychological assessments (Yuyama et al. 2014). The experimental and clinical evidence on the GM1 positive effect suggest a possible role not only for plasma membrane GM1 deficiency but also for AD pathogenesis that, in our opinion, should be investigated more in detail.

3.6.1.3 Huntington's Disease

Altered levels of glycosphingolipids content have been correlated to a third neurodegenerative disorder: HD (Desplats et al. 2007; Maglione et al. 2010; Ledeen and Wu 2018a; Magistretti et al. 2019). This condition, characterized primarily by involuntary movements and impaired motor coordination, is caused by an autosomal dominant mutation in the huntingtin (HTT) gene determining the expansion of poliQ stretch at N-terminus of the protein that results in HTT misfolding and aggregation (Magistretti et al. 2019).

By analyzing postmortem caudate HD samples, Desplats and coworkers identified a decreased expression of enzymes involved in ganglioside synthesis, including St3gal5, St8sia3, St3gal2, and B4galnt1 (Desplats et al. 2007). Consequently, an overall remodeling of ganglioside expression and a significant elevation of GD3 ganglioside accompanied by decreased level of GM1 were found (Desplats et al. 2007). The analyses of different HD transgenic mouse models confirmed the alteration of ganglioside pattern with a GM1 reduction in the striatum and cortex, two regions involved in HD (Desplats et al. 2007; Maglione et al. 2010), and a significant decrease of cerebrosides and sulfatides in the forebrain suggesting abnormalities in myelin content (Desplats et al. 2007).

In vitro administration of GM1 protected HD cells from apoptosis via PI3K/AKT pathway activation (Maglione et al. 2010) and, accordingly, the intraventricular infusion of GM1 was able to improve motor and anxiety behaviors and cognitive functions in HD mice models (Maglione et al. 2010; Alpaugh et al. 2017). In line with the phenotypic amelioration, different molecular events were modulated by GM1 exposure. Depending on the HD model used, GM1 administration led to reduction of ferritin levels (Alpaugh et al. 2017) that correlate to striatal and cortical atrophy in HD patients (Magistretti et al. 2019), to restoration of expression and phosphorylation of DARPP-32 protein involved in modulation of dopamine signaling. Additionally, GM1 treatment of HD model shows to modulate different neurotransmitters levels (Alpaugh et al. 2017), to attenuate HTT toxicity by inducing the phosphorylation at Ser13 and Ser16 that allow the decrease of HTT aggregates (Di Pardo et al.

2012) and by reducing both soluble and insoluble mutant HTT (Alpaugh et al. 2017).

3.6.1.4 Peripheral Neuroprotection

From 1976, gangliosides were used as commercial drug for the therapy of peripheral neuropathies, but at the beginning of the 1990s, gangliosides were claimed by the medical society as responsible for inducing the Guillain-Barré syndrome (Sonnino et al. 2017) and the drug was withdrawn in many countries. Today, the idea that the administration of gangliosides is not associated with the onset of Guillain-Barré syndrome seems to be sounder (Magistretti et al. 2019), and in recent years, clinical trials at different phases still involve GM1 ganglioside. One of these, related to the association of GM1 with oxaliplatin chemotherapy seems to give very promising results.

Oxaliplatin is a very powerful drug against gastrointestinal tumors; however, it is characterized by severe peripheral neurotoxicity. Sixty patients with gastrointestinal tumors were injected daily with 100 mg of GM1 for the 3 days following the chemotherapy treatment. The treatment showed some protection against the oxaliplatin neurotoxicity (Zhu et al. 2013).

3.6.2 Glycosphingolipidoses

For a long time, lysosomes have been considered the final destination of degradative pathways, but it is now clear that they are also crucial regulators of cell homeostasis and their impairment causes the onset of severe pathology called lysosomal storage disorders (LSDs) (Perera and Zoncu 2016).

LSDs are caused by a reduced or absent enzymatic activity of catabolic lysosomal enzymes, by defect in the nonenzymatic lysosomal activator proteins or in nonlysosomal proteins involved in glycohydrolases maturation. These defects result in the intralysosomal accumulation of undegraded metabolites that leads to the onset of cell damage (Platt et al. 2012).

Classically, LSDs are classified based on the nature of the accumulated substrate: mucopolysaccharidoses (accumulation of mucopolysaccharides), sphingolipidoses (sphingolipids), and oligosaccharidoses also known as glycoproteinoses (oligosaccharides) (Filocamo and Morrone 2011). More recently LSDs have also been classified by the molecular defect, including more pathologies recognized now as LSDs: (1) nonenzymatic lysosomal defects, (2) transmembrane protein defects (transporters and structural proteins), (3) lysosomal enzyme protection defects, (4) post-translational processing defects of lysosomal enzymes, (5) trafficking defects in lysosomal enzymes and (6) polypeptide degradation defects (Table 3.4).

LSDs clinical manifestations involve multiple organs and systems (Wang et al. 2011). The principal pathological phenotypes are represented by hepatosplenomegaly, corneal or lenticular opacities, retinal dystrophy, optic nerve atrophy, glaucoma, blindness, bone dysplasia, abnormalities of bone density, and osteonecrosis (Parenti et al. 2015). About two-thirds of patients affected by LSDs also show an important neurological deficiency, which is extremely variable and heterogeneous ranging from progressive neurodegeneration and severe cognitive deficit to psychiatric and behavioral disorders (Parenti et al. 2015). This is particularly true for lysosomal storage disorders caused by defects in SL metabolism (sphingolipidoses); reviewed in (Futerman et al. 2004; Futerman and Van Meer 2004; Kolter and Sandhoff 2006). The onset of symptoms can occur before the birth, for the most severe phenotypes, or during the adulthood for the late-onset mild forms. Severity and age of onset in LSDs depend by several factors including: residual enzyme activity, distribution of tissue-specific and cell-specific substrates, cell turnover rate, defective protein expression, and other mechanisms that influence the lifespan of affected cells (Jakóbkiewicz-Banecka et al. 2014).

The complexity of LSDs, at the phenotypic and molecular level, particularly considering the aspects related to neuronal dysfunctions, clearly indicates that, even if it is undoubtedly clear that the intralysosomal accumulation of unmetabolized substrates is the primary cause of the dis-

Table 3.4 Lysosomal storage disorders and secondary accumulated gangliosides

Disease	Defective protein	Primary acccumutared substrate	Secondary accumulated gangliosides
Mucopolysaccharidoses (MPS)			
MPS I (Hurler, Scheie, Hurler/Scheie)	α-Iduronidase	Dermatan sulphate, heparan sulphate	GM2, GM3
MPS II (Hunter)	Iduronate sulphatase	Dermatan sulphate, heparan sulphate	GM2, GM3
MPS III A (Sanfilippo A)	Heparan sulphamidase	Heparan sulphate	GM2, GM3
MPS III B (Sanfilippo B)	Acetyl α-glucosaminidase	Heparan sulphate	GM2, GM3
MPS III C (Sanfilippo C)	Acetyl CoA: α-glucosaminide *N*-acetyltransferase	Heparan sulphate	*NE*
MPS III D (Sanfilippo D)	*N*-acetyl glucosamine-6-sulphatase	Heparan sulphate	GM3
MPS IV A (Morquio A)	Acetyl galactosamine-6-sulphatase	Keratan sulphate, chondroiotin 6-sulphate	*NE*
MPS IV B (Morquio B)	β-Galactosidase	Keratan sulphate	*NE*
MPS VI (Maroteaux-Lamy)	Arylsulphatase B	Dermatan sulphate	GM3
MPS VII (Sly)	β-Glucuronidase	Dermatan sulphate, heparan sulphate, chondroiotin 6-sulphate	GM2, GM3
MPS IX (Natowicz)	Hyaluronidase	Hyluronan	*NE*
Sphingolipidoses			
Fabry	α-Galactosidase A	Globotriasylceramide	*NE*
Farber	Acid ceramidase	Ceramide	*NE*
Gangliosidosis GM1 (Types I, II, III)	GM1 β-galactosidase	GM1 ganglioside, Keratan sulphate, oligosaccharides, glycolipids	GM2, GM3
Gangliosidosis GM2 (Tay-Sachs)	β-Hexosaminidase A	GM2 ganglioside, oligosaccharides, glycolipids	GM3
Gangliosidosis GM2 (Sandhoff)	β-Hexosaminidase A + B	GM2 ganglioside, oligosaccharides, glycolipids	GM3
Gaucher (Types I, II, III)	Glucosylceramidase	Glucosylceramide	GM2, GM3, GM1, GD3
Krabbe	β-Galactosylceramidase	Galactosylceramide	*NE*
Metachromatic leucodystrophy	Arylsulphatase A	Sulphatides	*NE*
Niemann-Pick (Types A, B)	Sphingomyelinase	Sphingomyelin	GM2, GM3
Oligosaccharidoses (Glycoproteinoses)			
Aspartylglicosaminuria	Glycosylasparaginase	Aspartylglucosamine	*NE*
Fucosidosis	α-Fucosidase	Glycoproteins, glycolipids, fucoside-rich oligosaccharides	*NE*
α-Mannosidosis	α-Mannosidase	Mannose-rich oligosaccharides	*NE*
β-Mannosidosis	β-Mannosidase	Man(β1→4)GlnNAc	*NE*
Schindler	*N*-acetylgalactosaminidase	Sialylated/ asialoglycopeptides, glycolipids	*NE*
Sialidosis	Neuraminidase	Oligosaccharides, glycopeptides	*NE*
Glycogenoses			
Glycogenosis II/Pompe	Acid maltase	Glycogen	*NE*

(continued)

Table 3.4 (continued)

Disease	Defective protein	Primary acccumutared substrate	Secondary accumulated gangliosides
Lipidoses			
Wolman/CESD	Acid lipase	Cholesterol esters	*NE*
Non-enzymatic lysosomal protein defect			
Gangliosidosis GM2, activator defect	GM2 activator protein	GM2 ganglioside, oligosaccharides	/
Metachromatic leucodystrophy	Saposin B	Sulphatides	*NE*
Krabbe	Saposin A	Galactosylceramide	*NE*
Gaucher	Saposin C	Glucosylceramide	GM2, GM3, GM1, GD3
Transmembrane protein defect			
Transporters			
Sialic acid storage disease; infantile form (ISSD) and adult form (Salla)	Sialin	Sialic acid	*NE*
Cystinosis	Cystinosin	Cystine	*NE*
Niemann–Pick Type C1	Niemann–Pick type 1 (NPC1)	Cholesterol and sphingolipids	GM2, GM3, GM1
Niemann–Pick, Type C2	Niemann–Pick type 2 (NPC2)	Cholesterol and sphingolipids	GM2, GM3
Structural Proteins			
Danon	Lysosome-associated membrane protein 2	Cytoplasmatic debris and glycogen	*NE*
Mucolipidosis IV	Mucolipin	Lipids	*NE*
Lysosomal enzyme protection defect			
Galactosialidosis	Protective protein cathepsin A	Sialyloligosaccharides	GM2, GM3, GM1, GD1a
Post-translational processing defect			
Multiple sulphatase deficiency	Multiple sulphatase	Sulphatides, glycolipids, GAGs	*NE*
Trafficking defect in lysosomal enzymes			
Mucolipidosis IIα/β, IIIα/β	GlcNAc-1-P transferase	Oligosaccharides, GAGs, lipids	GM1
Mucolipidosis IIIγ	GlcNAc-1-P transferase	Oligosaccharides, GAGs, lipids	Multiple
Polypeptide degradation defect			
Pycnodysostosis	Cathepsin K	Bone proteins	*NE*
Neuronal ceroid lipofuscinoses (NCLs)			
NCL 1	Palmitoyl protein thioesterase	Saposins A and D	*NE*
NCL 2	Tripeptidyl peptidase 1	Subunit c of ATP synthase	*NE*
NCL 3	CLN3, lysosomal transmembrane protein	Subunit c of ATP synthase	*NE*
NCL 5	CLN5, soluble lysosomal protein	Subunit c of ATP synthase	*NE*
NCL 6	CLN6, transmembrane protein of ER	Subunit c of ATP synthase	*NE*
NCL 7	CLC7, lysosomal chloride channel	Subunit c of ATP synthase	*NE*
NCL 8	CLN8, transmembrane protein of ER	Subunit c of ATP synthase	*NE*
NCL 10	Cathepsin D	Saposins A and D	GM2, GM3

NE not evaluated; data were collected from (Walkley 2004; Walkley and Vanier 2009)

ease, other, still obscure, molecular mechanisms might lead from this event to the pathology.

Since SL metabolism and traffic is a complex network of interdependent events, and the recycle of catabolic fragments originated in the lysosome for biosynthetic purposes is quantitatively relevant, it can been expected that the blockade of proper sphingolipids catabolism at the lysosomal level leads to the jamming of the overall flow of metabolites, with consequences on the sphingolipids composition in all cellular districts, including the plasma membrane. Interestingly, in confirmation of this, more than 70% of LSDs is characterized by the secondary accumulation of glycosphingolipids that is not confined to the lysosome but also occurs at the plasma membrane level.

The cell machinery responsible for cell sphingolipids pattern is complex and still not completely understood in its details. The Golgi de novo biosynthesis, that is necessary to replace the molecules catabolized in the lysosomes as the consequence of membrane turnover, is considered the heart of the metabolic process, and is regarded as the main regulatory site. Nevertheless, in recent years many other processes capable to modulate the final PM composition have been included to the complex sphingolipids biosynthetic pathway: (1) partially catabolized compounds can leave the lysosomes and can be transported to the Golgi for reassembling or to any other membrane (Kitatani et al. 2008); (2) both sphingolipids synthases and hydrolases are associated with the PM allowing changes of the content of ceramide, SM, and gangliosides directly in situ; (3) PM sphingolipids can be shed in the extracellular milieu ad can be partially reassociated with the same membrane or associated with the membranes of neighboring cells; (4) intracellular vesicles (endosomes, lysosomes, exosomes, and other vesicles) can fuse with PM; and (5) lipids, including sphingolipids, can undergo active trafficking between different intracellular membranes mediated by membrane contact sites (MCSs).

Interestingly, using an artificial model of lysosomal impairment represented by human fibroblasts loaded with sucrose, it has been described that the impaired lysosomes are not able to catabolize the sphingolipids leading to their lysosomal accumulation (Samarani et al. 2018). Nevertheless, as already demonstrated in a cellular model of Niemann Pick type A disease (Gabandé-Rodríguez et al. 2014), it has been found that the SL accumulation not only is confined to lysosomes but also affects the PM. The molecular mechanism responsible for the migration of noncatabolized sphingolipids from lysosomes to the PM is not understood so far, even if several line of evidence support the activation of two mechanisms: (1) the intralysosomal vesicles accumulating SL could exchange monomers with the internal leaflet of the lysosomal membrane that, by the fusion with the cell surface, becomes component of the PM; (2) the lysosomal exocytosis may induce the release in the extracellular milieu of the intralysosomal vesicles accumulating sphingolipids that, by uptake, could be inserted into the outer leaflet of the cell membrane. This study points out also on the importance of the PM sphingolipids alteration in determining the onset of cell damage in lysosomal storage disorders that is independent by the primary accumulated substrate due to the genetic defect.

Indeed, in LSDs the activation of the lysosomal exocytosis not only is responsible for an increased content of glycosphingolipids but drives also lysosomal hydrolases at the external leaflet of the PM. The concomitant aberrant presence of glycosphingolipids and its hydrolases induces conformational changes in the membrane architecture that are responsible for the onset of cells damage (Samarani et al. 2018) (Fig. 3.14).

The bioactive role of PM sphingolipids is mainly related to the lipids-rafts concept. The lateral segregation of sphingolipids, and particularly of gangliosides, is considered a major driving force for the formation of lipid domains (Simons and Ikonen 1997; Sonnino et al. 2006; Sonnino and Prinetti 2013), where they modulate the activity of a number of cell surface receptors and enzymes (Simons and Toomre 2000; Prinetti et al. 2009). The resulting sphingolipids-enriched membrane domains with non-physiological com-

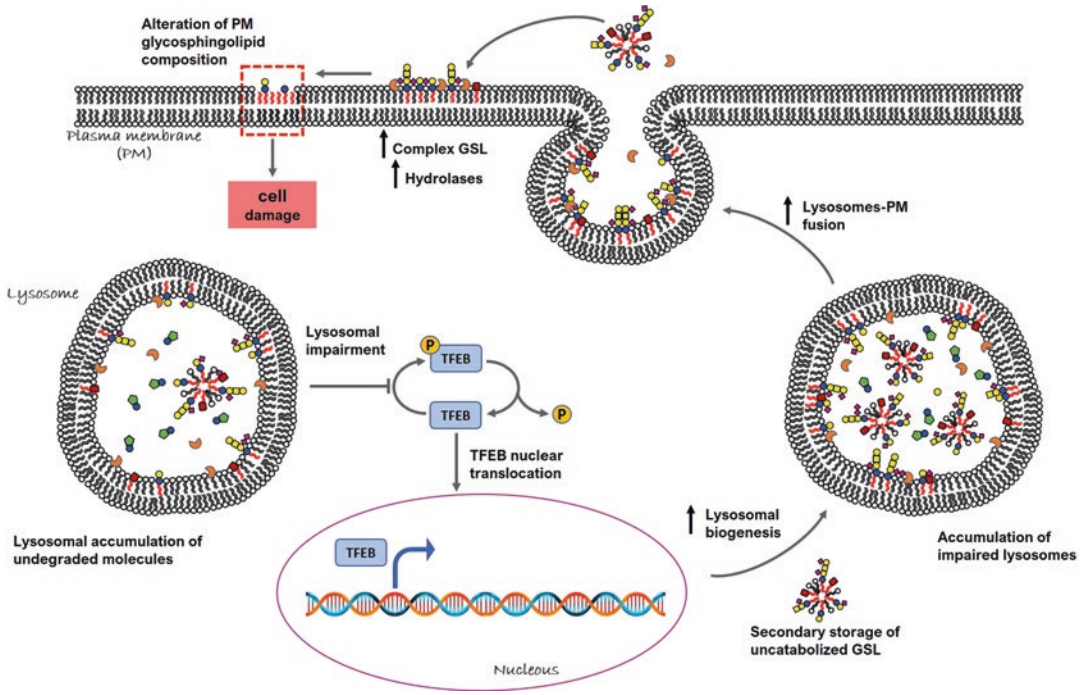

Fig. 3.14 Diagram showing the lysosome-PM axis suggested to explain the mechanism linking lysosomal storage and the onset of cell damage resulting in cell growth arrest

position might be responsible for altered signaling events involved in the onset of the cellular damage and of tissue pathology.

This hypothesis has been recently confirmed by several observations: (1) in a cell model of GD, impaired lysosomal catabolism of GlcCer led to the accumulation of GlcCer at the plasma membrane level in lipid rafts, possibly explaining the altered lipid and protein sorting observed in this pathological condition (Hein et al. 2008); moreover, it has been reported that GD is associated with insulin resistance (Langeveld et al. 2008). Since insulin receptor function is regulated by its interaction in lipid rafts with GSL (Kabayama et al. 2007) and in particular, GM3 ganglioside, this suggests that the altered lipid rafts' organization in Gaucher cells might be responsible for altered responsiveness to insulin; (2) psychosine (galactosylsphingosine) is one of the galactoslylsphingolipids that accumulates in the brain of Krabbe disease (human globoid cell leukodystrophy) patients due to the deficient activity of β-galactosylceramidase. Psychosine accumulates in lipid rafts from brain and sciatic nerve from twitcher mice (the animal model for the infantile variant of the disease) and from human Krabbe patients, leading to an altered distribution of lipid raft proteins and to inhibition of protein kinase C (White et al. 2009); (3) in brains from ASMKO mice, an animal model for Niemann–Pick disease type A (due to deficient activity of the lysosomal acid sphingomyelinase) (Schuchman 2007), in addition to the expected SM accumulation, we observed an unexpected remodeling of the fatty acid composition of the accumulated SM and a significant increase in ganglioside content, mainly due to the accumulation of monosialogangliosides GM3 and GM2, leading to a non-conventional lipid raft organization (Scandroglio et al. 2008; Buccinnà et al. 2009). Taken together, these observations open a new scenario on the existence of a lysosome-PM axis fundamental to maintain the cell homeostasis that in case of alterations is responsible for the onset of cell damage.

Starting from the '90, the enzyme replacement therapy has been introduced for treatment of glycogen storage disease type II, the Pompe disease caused by a deficiency of the lysosomal acid α-glucosidase, of a few several mucopolysaccharidoses and a few glycosphingolipidoses. Nevertheless, the treatment could not generally be applied to the LSDs with serious damage of the central nervous system due the obstacle posed by the BBB (Li 2018; Concolino et al. 2018).

Administration of glucocerebrosidase to type 1 GD, that causes damages to liver, spleen, kidney, lung and bone, increases the activity of this enzyme intracellularly, and ameliorates symptoms. Several recombinant approved forms of glucocerebrosidase are available reporting modification of the glycosylation sites to have mannose at the end of the chain. This is necessary, to be recognized by mannose receptors present on the surface of macrophages and be then taken up. Side effects are mild, but present, such as, diarrhea, back pain, and many others (Revel-Vilk et al. 2018).

A similar approach has been used in building an α-galactosidase necessary to treat the Fabry disease, in which the enzyme deficiency in the lysosomal leads to the accumulation of globotriaosylceramide, with progressive damage of kidney and other organs, and to peripheral neuropathy.

Long-term treatment slows down disease progression, but many complications develop in most patients (Ortiz et al. 2018). Therefore, several new treatment approaches are under development and study: chaperone therapy aimed to reduce the catabolism of incorrect enzyme and increase its availability into the lysosomes; administration of the enzyme mRNA to help stimulate production of the enzyme; substrate reduction therapies by administration of inhibitors of glucosylceramide synthase.

3.6.3 Cancer

The origin and progression of tumors are characterized by several steps and features, including limitless replicative potential (the result of the combination of different factors, such as self-sufficiency in growth signals on one hand, and insensitivity to growth-inhibitory signals associated with escape from programmed cell death on the other hand), sustained angiogenesis, interplay with immune system cells, and eventually, in some cases, tissue invasion and metastasis. The initial events that trigger tumorigenic transformation are digging deeply into the genome (e.g. genetic mutations of oncogenes and tumor suppressor genes) of the cells undergoing transformation. On the other hand, in all the steps leading to cancer disease progression tumor-host interactions are of crucial importance. In the interactions between tumor cells and the surrounding microenvironment, the relevant interface is represented by the cellular surfaces. The surface of the tumor cell, the site where interactions between the cell and the extracellular environment are organized and transduced into signals able to modify the cell properties influencing the tumor phenotype. But also the surface of the incredible variety of cells present in the tumor stromal compartment, including endothelial cells, fibroblasts, and resident and recruited inflammatory and immune system cells. Thus not surprisingly, the glycocalyx of tumor cells is characterized by multiple and complex alterations, and the altered expression of carbohydrate epitopes at the tumor cell surface ("aberrant glycosylation") is involved in many, if not all, of the steps of tumor progression. The term "aberrant glycosylation" indicates an altered expression of oligosaccharide epitopes, associated with both glycolipids and glycoproteins. Independent reports from several authors, but in particular the extensive work in this area of Dr. Sen-itiroh Hakomori and his scholars allowed to define aberrant glycosylation as a general feature of human cancer (Hakomori 1985), and to elucidate the main metabolic alterations responsible for aberrant glycosylation in cancer. At least two different metabolic mechanisms contribute to the generation of tumor-associated carbohydrate epitopes: (1) the impairment of specific glycosylation steps ("incomplete synthesis"); (2) the induction of genes encoding for glycosyltransferases or, less frequently, for carbohydrate transporters ("neo-

synthesis") (Hakomori 1996). As the result, tumors are characterized by the appearance and/or accumulation of carbohydrate epitopes that are often present in tumor-associated antigens. Indeed, glycan epitopes associated with aberrant glycosylation in tumors were in most cases originally defined by their ability to raise the production of specific antibodies, and only later their molecular structures have been characterized. The discovery of oligosaccharide tumor-associated antigens and the development of different antibodies reacting against them provided useful diagnostic and research tools, and opened the field of tumor glycobiology, that developed tremendously in the following decades.

As already mentioned, aberrant glycosylation in tumors do affect carbohydrate epitopes associated with both glycolipids and glycoproteins. Sphingolipids, and in particular glycosphingolipids, mediate several aspects of the interactions between a cell and the extracellular environment or other cells under physiological conditions. They are known as cell surface antigens, as mediators of cell-cell recognition and cell adhesion and as modulators of several aspects of signal transduction processes involved in cell survival/proliferation and differentiation. Aberrant glycosylation in GSL seems to play very relevant roles under pathological conditions, and several lines of evidence point on the importance of aberrantly expressed GSL in the formation and progression of tumors. Indeed, the notion that GSL metabolism and expression is dramatically changed during neoplastic transformation and tumor progression was probably the origin of the concept of aberrant glycosylation associated with tumors, and it is rooted into multifaceted experimental evidence. In several tumors, total ganglioside-bound sialic acid levels was found to be higher than in the corresponding normal tissue (e.g., in breast tumor tissues v. normal mammary tissues (Marquina et al. 1996). The chemical structure of sialic acid associated with tumor tissue gangliosides was different in several tumors, in particular gangliosides containing *N*-glycolylneuraminic acid, usually expressed at low levels in humans, were relatively abundant (Hakomori 1989) (this finding led to the hypothesis that consumption of foods rich in *N*-glycolylneuraminic acid could favor the onset of certain tumors). Moreover, the accumulation of high levels of specific gangliosides and/or neutral GSLs in specific types of cancer was reported: this is the case for GD3, GD2 and GM3 gangliosides in human and mouse melanoma, GD2 in neuroblastoma, Gg3 in human and mouse lymphoma, fucosyl-GM1 in small cell lung carcinoma, globo-H in breast and ovarian carcinoma, disialosylgalactosylgloboside in renal cell carcinoma. Several pieces of evidence pointed out that aberrant glycosphingolipid expression is not simply an epiphenomenon accompanying neoplastic transformation, but rather suggested that aberrant GSL expression could actively contribute to the tumor phenotype. In vitro, it has been shown that tumor cell lines with higher tumorigenic potential are characterized by higher ganglioside levels (Ladisch et al. 1987; Deng et al. 2000), and that the artificially induced increase in cellular ganglioside levels enhanced the ability to form experimental tumors. On the other hand, in human cancer patients correlations between the expression levels of some GSL structures and tumor malignancy and/or patient survival rates have been observed (e.g. disialosylgalactosylgloboside in renal cell carcinoma (Satoh et al. 1996), galactosylgloboside in seminoma (Ohyama et al. 1996) and GM3 ganglioside in bladder cancer – the latter example will be further discussed in this section) (Hakomori 1996, 2002). Another intriguing aspect of aberrant GSL behavior in tumors is represented by the ability of tumor cells to shed significant amounts of selected ganglioside species mostly in the form of vesicles (Ladisch et al. 1994, 1997; Chang et al. 1997). GSL shedding from cells is known for long time and is also present in non-tumor cells, however tumor cells do shed much higher amounts of GSL, and, as mentioned, there is apparently a selection of the molecular species that are preferentially shed. Many Authors argued that GSL shedding might reflect a non-physiological membrane turnover typical of cultured cells, however GSL and in particular gangliosides are found in human serum, and, remarkably, serum ganglioside levels

in cancer patients were found higher than in healthy individuals (Ladisch et al. 1994, 1997; Chang et al. 1997). When GSL shedding was discovered, researchers still had a quite naïve view about the interplay between the tumor and the immune system, that is indeed very complicated and crucial for the progression of the tumor itself. At that time, it has been hypothesized that tumor-derived gangliosides released in the host environment might play a role in eluding the aggression by the host immune system. Now it is becoming evident that release of selected ganglioside species from tissues characterized by chronic low-grade inflammation (a feature considered a tumor hallmark) do interact with specific immune system cells subpopulations, and in particular do affect the function of innate immune response, likely contributing to the progression of TLR4-mediated diseases, possibly including some forms of cancer (Kanoh et al. 2020).

The physiological roles of GSL in modulating cell properties are multiple and at least in part highly specific for a given GSL molecular species. Thus, not surprisingly, the consequences of aberrant GSL expression on tumor cell phenotype are as well multifaceted. The modification of glycosphingolipid expression deeply affects several properties of tumor cells, that are directly relevant to the growth and progression of the tumor, and to metastasis formation: cell proliferation/survival, cell adhesion (to other cells in the original tumor mass for solid tumors, to the extracellular matrix or to the endothelium of blood vessels), motility, recognition and invasion of host tissues. This heterogeneity at the cellular level is reflected by the corresponding heterogeneity in the underlying interactions at the molecular levels.

At the molecular level, the phenotypic alteration in tumor cells explained most straightforwardly by aberrant GSL expression is probably the proliferation sustained by the interaction of GSL, in particular gangliosides, with classical tyrosine kinase growth factor receptors. The ability of GSL, and gangliosides in particular, to modulate the activity of receptor tyrosine kinases, has been widely documented (Hakomori and Igarashi 1995; Yates and Rampersaud 1998). Still to be elucidated in most cases remain the molecular aspects of GSL-protein interactions underlying the modulatory effect of GSL. However, at least for some growth factor receptors, the interaction with gangliosides is facilitated by the co-clustering of the receptor itself and of the interacting lipids within lipid rafts. The sustained proliferation of tumor cells is supported by a network of different soluble growth factors produced from different cells present or recruited in the tumor stromal compartment. Among those, the EGFR is particularly relevant, and prognosis of patients affected by several solid tumors (for example, small cell lung carcinoma) is determined by mutations in the EGFR. EGFRis effectively inhibited by GM3 (and, at lesser extent, by other gangliosides) (Bremer et al. 1986). GM3 inhibited receptor autophosphorylation but not receptor dimerization (Zhou et al. 1994), neither its binding with the ligand, EGF (Yednak and Bremer 1994). The sialyllactose oligosaccharide is essential for ganglioside-receptor interaction (Miljan et al. 2002), being involved in side-by-side carbohydrate-carbohydrate interactions with *N*-linked glycan bearing multiple GlcNAc terminal residues on the receptor (Yoon et al. 2006a, b). GM3/EGFR interaction is facilitated by the enrichment of EGFR in GM3-enriched lipid rafts (Ringerike et al. 2002; Roepstorff et al. 2002). However, it is becoming clear that different subpopulations of EGFR associated with distinct membrane domains exist. Interestingly, high levels of GM3 seem to be able to negatively regulate EGF-dependent proliferation of tumor cells with other mechanism, in addition to the direct inhibition of the receptor as described above. In some cell types, GM3 overexpression favors the interaction of caveolin-1, and important modulator of tumor phenotype, with EGFR, in turn causing inhibition of EGFR tyrosine phosphorylation and dimerization (Wang et al. 2002). Thus, GM3 influences EGFR signaling by a second molecular mechanism, clearly distinct from the direct inhibition, by modulating EGFR/caveolin-1 association.

The modulation of cell adhesion, motility and recognition seems to be modulated through a wide variety of GSL-binding proteins and/or

GSL-GSL interactions (Hakomori 1996, 2002; Hakomori et al. 1998). In particular, GSL might contribute to the modulation of integrin-dependent interactions of tumor cells (determining their adhesion, motility and invasiveness, but also affecting their proliferation potential) with the extracellular matrix as well as with host cells present in the stromal compartment of the tumor. GSL might also be involved in selectin- or galectin-dependent adhesion of tumor cells to endothelial cells, a crucial step in the extravasation of circulating tumor cells and in the initiation of metastasis. At least in some cases, *trans* GSL-GSL interactions are also important in determining the motility and metastatic potential of tumor cells, while in others GSL at the tumor cell surface have anti-adhesive properties. For example, GM3-GM3 interaction-mediated repulsion of tumor cells was implicated in the release of cells from the tumor mass, playing a role in initiation of metastasis. On the other hand, adhesion of circulating melanoma cells to endothelial cells, a critical step in melanoma cell metastasis, was mediated by the heterophilic trans interaction between GM3 at the surface of melanoma cells and lactosylceramide and/or Gg3 at the surface of endothelial cells. Melanoma cells usually do express high levels of GM3, while in turn endothelial cells express LacCer and Gg3. In vitro, GM3-rich B16 melanoma cells were able to adhere to LacCer- or Gg3 coated dishes. GM3-dependent adhesion is probably the best documented example of trans head-to-head carbohydrate-carbohydrate interaction. Remarkably, GM3-dependent adhesion (in a similar way to integrin-dependent adhesion) is not merely a physical link. B16 adhesion mediated by GM3-LacCer or GM3-Gg3 interactions and led to initiation of signal transduction, in particular FAK activation and enhanced GTP loading on Rho and Ras, with consequent phenotypic changes in terms of motility and morphology, suggesting that B16 GM3-dependent adhesion is associated with enhanced B16 cell motility and thereby initiates metastasis (Kojima et al. 1992; Iwabuchi et al. 1998). In these cells, GM3 is closely associated with signaling proteins such as c-Src, Rho and Ras within sphingolipid-enriched membrane domains, and binding with Gg3 or anti-GM3 antibody stimulates focal adhesion kinase phosphorylation and c-Src activity. Activation of c-Src and FAK with enhanced motility and invasiveness was induced in MCF-7 breast carcinoma cells by anti monosialyl-Gb5 monoclonal antibody, but not by antibodies to other glycosphingolipids (anti-Gb3, anti-Gb5, anti-GM2) (Steelant et al. 2002).

Recently, it has been proposed that many aspects of tumor cell social life are mediated by cell surface signaling complexes regulated by GSL. Again, lipid rafts seem to play a relevant role in the organization of these signaling complexes, favoring the clustering of different interactors, made possible only when certain GSL are expressed above a given quantitative threshold. Apparently, highly hydrophobic adapter membrane proteins such as tetraspanins or caveolins are essential for the organization of these complexes. On the other hand, their function seems strictly dependent on their GSL composition, providing clues about the molecular mechanisms underlying the phenotypic effects associated with aberrant glycosylation.

In the case of GM3 ganglioside, a number of studies have shown that its expression levels do affect the tumor phenotype in multiple ways, in particular by controlling of tumor cell motility, invasiveness and survival. As mentioned above, in the case of GM3-dependent adhesion of melanoma cells, it has been shown that GM3 is closely associated with c-Src, Rho and Ras within Triton X-100 insoluble ganglioside-rich lipid rafts and binding to Gg3 triggers the phosphorylation of the focal adhesion kinase and stimulates c-Src activity (Iwabuchi et al. 1998). A similar functional association between a sialoglycolipid and c-Src and other related signaling molecules was observed for GM3 also in neuroblastoma cells (Prinetti et al. 1999), for disialylgalactosylgloboside in renal carcinoma cells (Satoh et al. 2000), and for monosialyl-Gb5 in breast carcinoma cells (Steelant et al. 2002).

In bladder cancer, GM3 is highly expressed in non-invasive, superficial tumors compared with invasive tumors, as result of the upregulation of relevant glycosyltransferases (Satoh

et al. 1996; Satoh et al. 2001; Kawamura et al. 2001). In a non-invasive cell line (KK47) originated from superficial human bladder cancer, GM3 levels were higher than in the invasive YTS1 human bladder cancer cell line. In this kind of tumor, the regulation of adhesion/motility by GM3 requires a multimolecular signaling complex organized by members of the tetraspan membrane protein superfamily ("tetraspanins"). Tetraspanins are highly hydrophobic integral membrane proteins strongly interacting with GSL (indeed, they were originally defined as "proteolipids") (Kawakami et al. 2002), and the best characterized member, CD9, has been frequently described as interacting with integrin receptors (Hemler 1998). Tetraspanin CD9 and integrin α3 or α5 are associated within the same Brij 98-insoluble glycolipid-enriched domain. However, the physical and functional interaction between the tetraspanin and integrins, and the resisting downstream signaling controlling cell motility, are strongly affected by the cellular levels of GM3. In fact, knock down of CD9 or GM3 depletion in non-invasive KK47 cells induced their phenotypic conversion into invasive variants. On the other hand, exogenous GM3 addition induces the phenotypic reversion of the highly invasive and metastatic cell lines YTS1 to low motility variants. The changes in cell motility observed upon manipulation of GM3 levels were strictly correlated with the association of CD9 with α3 integrin. The level of interaction was positively modulated by GM3 and was thus higher in non-invasive than in highly invasive cells. In turn, CD9/α3 integrin association was reduced by GM3 depletion in KK47 and conversely enhanced by exogenous GM3 addition in YTS1 cells.

GM3 levels in these cells control not only CD9/α3 integrin association, but also the downstream signaling events by controlling the activation state of c-Src. The activity of the non-receptor tyrosine kinase c-Src is usually very high in malignant, invasive tumors, where c-Src is frequently constitutively overexpressed. The extendt of c-Src association to the CD9/α3 complex is negatively determined by the cellular GM3 levels. Moreover, with a still unclear mechanism, GM3 levels determine the extent of translocation and recruitment of the kinase Csk to the CD9/α3/c-Src complex. C-Src phosphorylation by Csk represents the main inhibitory mechanism for c-Src activity. c-Src is present in higher amount in the glycosynapse fraction in YTS1 cells, and it is activated in cells with low GM3 levels and high invasive potential (YTS1 or GM3-depleted KK47). On the other hand, exogenous addition of GM3 to YST1 cells caused Csk translocation to the lipid raft fraction and consequent inactivation of c-Src, influencing cell motility (Mitsuzuka et al. 2005).

Thus, GM3 complexed with CD9 and integrin receptors seems to play a crucial role in the control of tumor cell motility and invasiveness. This role of CD9/GM3 complexes in the regulation of integrin-mediated cell adhesion and signal transduction in oncogenic transformation, was confirmed by experiments showing the effect of the manipulation of GM3 levels in transformed cells. v-Jun-transformed mouse and chicken embryo fibroblasts were characterized by lower GM3 levels and down–regulated GM3 synthase mRNA levels respect to the non-transformed counterparts (Miura et al. 2004) and reversion of v-Jun oncogenic phenotype could be achieved by enhanced GM3 synthase gene transfection. During phenotypic reversion of v-Jun-transformed cells induced by GM3 synthase transfection, the association of CD9 and integrin receptors complex was increased.

Most notably, artificially increasing cellular GM3 levels (by loading cells with exogenous GM3 (Kawamura et al. 2001) or by pharmacological treatments (Satoh et al. 2001; Nojiri et al. 2002) led to a strong reduction of the tumorigenic activity and/or of the invasive potential of different human tumor cells lines. Consistently with this observation, the stable overexpression of GM3 synthase (SAT-I) in a mouse bladder carcinoma cell line reduced cell proliferation, motility and invasion with concomitant increase in the number of apoptotic cells (Watanabe et al. 2002). High expression levels of GM3 with concomitant expression of the tetraspanin CD9 inhibited cell motility in colorectal (Ono et al. 1999, 2001) cancer cells, and in CHO mutants the coexpression of CD9 and GM3 is essential for the downregulation of cell motility.

All these data support the notion that altered ganglioside levels can affect the tumor phenotype in terms of adhesion/motility by regulation the organization and function of the integrin signaling machinery. Recently it has been shown that integrin signaling can be controlled by other kinds of GSL/tetraspanin complexes. GM2 ganglioside complexed with a different tetraspanin, CD82, inhibits the activation of Met tyrosine kinase induced by hepatocyte growth factor (Todeschini et al. 2007). This suggests that ganglioside-controlled integrin signaling complexes might be a general paradigm controlling cell motility via tyrosine kinase signaling.

Compliance with Ethical Standards

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Ethical Approval This article does not contain any studies with animal/human participants performed by any of the authors.

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Glycosaminoglycans

4

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Abstract

Glycosaminoglycans (GAGs) are important constituents of human glycome. They are negatively charged unbranched polysaccharides that are usually covalently attached to proteins, forming glycan–protein conjugates, called proteoglycans. Glycosaminoglycans play critical roles in numerous biological processes throughout individual development and are also involved in the pathological processes of various diseases. Based on their remarkable bioactivities and their universal involvement in disease progression, GAGs are applied as therapeutics or are being targeted or used in treating diseases. In this chapter, we introduce the characteristics of the four classes of GAGs that constitute the glycosaminoglycan family. The pathological roles of glycosaminoglycans in major diseases including innate disease, infectious disease, and cancer are discussed. The application of GAGs and their mimetics as therapeutics is introduced, as well as those therapeutic methods developed based on GAGs' role in pathogenesis. In addition, we provide a brief and overall lookback at the history of GAG research and sort out some critical techniques that facilitated GAG and glycomics studies.

Keywords

Glycosaminoglycans · Glycomics · Heparin · Innate disease · Infectious disease · Cancer

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4.1 Glycosaminoglycans: An Essential of the Human Glycome

Studies of the "-ome" or "-omic" burst onto the scene late in the last century and began to boom as the dramatic Human Genome Project was completed, revealing the complete genomic composition of humans, as well as a variety of other species. As with many scientific explorations, the

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outcome of the Human Genome Project resulted in many new and interesting questions. The project revealed that there were about 20,500 human genes (currently there are an estimated 30,000 genes), which was significantly fewer than previous estimates (50,000–140,000) (National Human Genome Research Institute 2018, 2020). But what seemed to be confusing was that humans are the most complicated creatures on earth while having only slightly more genes than found in a nematode worm (*Caenorhabditis elegans*, with 19,427 genes predicted in 2003) (Kamath et al. 2003). Diverse post-translational modifications of proteins serve as an explanation for this dilemma. Glycosylation is one of the most important post-translational modifications of proteins. More than 50% of proteins in humans are glycosylated (Wong 2005). Glycans are covalently linked to protein scaffolds through catalysis involving glycosyltransferases, generating various glycosylated molecules to fulfill numerous biological functions.

The total collection of glycans synthesized by a cell, tissue, or organism under the specified conditions of time, space, and environment is termed the "glycome" (Varki et al. 2017). Most glycans in the human body are covalently attached to proteins or lipids. Glycan-protein conjugates can be categorized as glycoproteins or proteoglycans. Glycoproteins consist of two major groups of branched glycans, *N*-linked and *O*-linked glycans, attached to the protein scaffold to asparagine and serine/threonine, respectively (Miyoshi et al. 2020). Another group of glycan-protein conjugates are the proteoglycans (PGs), consisting of a core protein carrying one or more long carbohydrate structures, called glycosaminoglycans (GAGs).

GAGs are heterogeneous, negatively charged, unbranched polysaccharides that are usually covalently attached to proteins, forming PGs. Most PGs also contain *N*- and *O*-glycans that are typically found in glycoproteins. However, the GAG chains are more distinctive as they are usually much larger than these other types of glycans. For example, a 20 kDa GAG chain contains approximately 80 sugar residues, whereas a typical biantennary *N*-glycan contains 10–12 residues (Lindahl et al. 2017). GAGs and PGs are present on the cell surface and along with other glycans constitute the "glycocalyx." GAGs and PGs are also present in the intracellular milieu, and in extracellular matrix (ECM) as well (Fig. 4.1). GAGs comprised a repeating disaccharide unit. Each disaccharide building block consists of an amino sugar (i.e., *N*-acetylglucosamine [GlcNAc], or *N*-acetylgalactosamine [GalNAc]), and a uronic acid (i.e., D-glucuronic acid [GlcA], or L-iduronic acid [IdoA]) or D-galactose. These disaccharide units can be *O*- or *N*- substituted with sulfo groups at different positions. GAGs have been classified into four different families based on the form of their component sugar: heparin/heparan sulfate (HP/HS); chondroitin/dermatan sulfate (CS/DS); keratan sulfate (KS); and hyaluronan (HA). Except for HA, GAGs from the other three families are all conjugated to core proteins as PGs and are synthesized principally in the Golgi apparatus of cells (Fig. 4.1c). The HA is synthesized at the cellular plasma membrane and is unlinked to a core protein. In the extracellular space, HA is typically non-covalently bound to matrix proteins and to some PGs (Fig. 4.1a, b).

In a PG, the core protein is synthesized by a template-driven process as defined by the sequence of nucleotides in the corresponding gene. In contrast, GAGs are biosynthesized in a highly complex, template-free process catalyzed by specific enzymes (Chatterjee et al. 2019). In the case of HS, for example, 26 enzymes that participate in its biosynthesis have been identified. These enzymes catalyze processes such as *N*-deacetylation, *N*- and *O*-sulfation at different positions, and C5-epimerization of GlcA to IdoA (Soares da Costa et al. 2017). The different core proteins carrying GAGs are relatively few. There are approximately 17 with HS chains, approximately 20 with CS/DS chains, and approximately 8 with KS chains (Lindahl et al. 2017). PGs are considered among the most structurally complex glycoconjugates. The GAGs attached to a core protein can be in different types and numbers, and located at different

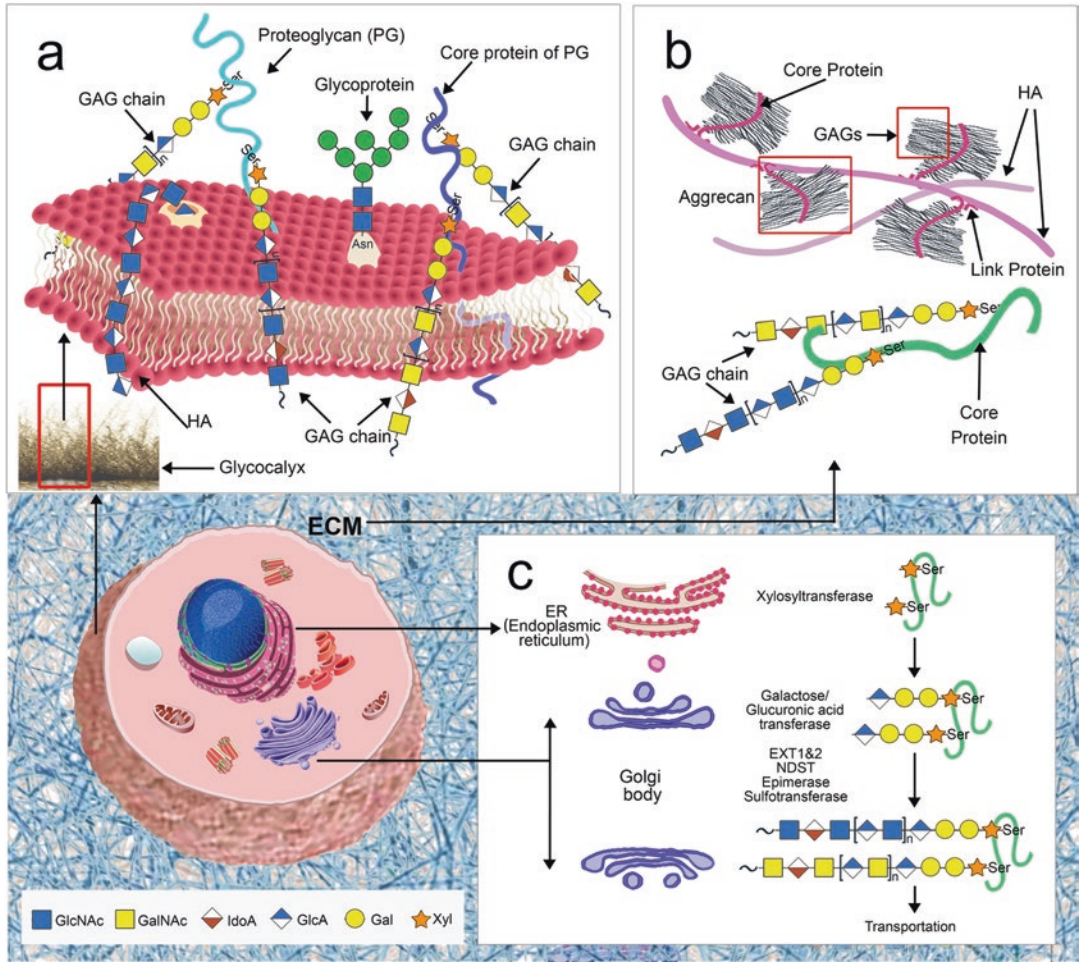

Fig. 4.1 A schematic diagram shows glycosaminoglycans (GAGs) and proteoglycans (PGs) on cell surface (**a**), in extracellular matrix (ECM), and biosynthesis of GAGs in cells (**c**). a. PGs, GAGs, and a glycoprotein, constituents of "glycocalyx" exhibiting on cell surface, and a hyaluronan (HA) chain being extruded through the membrane; (**b**) Some component proteoglycans in extracellular matrix, including proteoglycans carrying GAG chains and hyaluronan (HA) with/without aggrecan bound; (**c**) Biosynthesis of GAGs in endoplasmic reticulum and Golgi bodies catalyzed by a variety of enzymes

sites. The GAG chains also show a great diversity in monosaccharide composition, sequence, and hydroxyl group modifications. GAGs bind to many different classes of proteins mostly through electrostatic interactions between negatively charged sulfate and carboxylate groups of the GAG and positively charged amino acids of the protein (Esko et al. 2017). Through binding to their receptors, GAGs and PGs are able to carry out numerous biological functions and participate in various pathological processes.

4.2 Members of the Glycosaminoglycan Family

4.2.1 Heparan Sulfate and Heparin

HP/HS are bioactive GAGs that are *O*-glycosidically linked to serine residues of the core protein through a tetrasaccharide linkage region. The core protein associated with HP is serglycin. HP is synthesized on serglycin in mast

cells or basophils. The original molecular weight of the HP on GAG on its core protein is 60–100 kDa, but these HP chains are cleaved to 5–25 kDa and stored in secretory granules before being released into extracellular space (Köwitsch et al. 2018). HS is more widely distributed than HP. It can be assembled as cell-surface PGs, such as glypicans and syndecans, or pericellular PGs, such as perlecan and agrin.

The disaccharide building blocks of HP/HS family of GAGs are →4) α-L-IdoA/β-D-GlcA (1 → 4) α-D-GlcNS/-D-GlcNAc (1→. In the biosynthesis of HP, >70% of the GlcA residues undergo epimerization and are converted to IdoA. The IdoA in HP is generally 2-*O*-sulfated and the glucosamine can be *N*- and/or 6-*O*-sulfated. In HS, GlcA is the major hexuronic acid and HS generally contains only one sulfo group per disaccharide repeating unit, on 6-OH, -NH or 3-OH of the glucosamine residue, or the 2-OH of the uronic acid residue (Zhao et al. 2020). Notably, the degree of sulfation of HP is considerably higher than that of HS, due to extensive sulfate modification during its biosynthesis. HP is known as the most negatively charged biological macromolecule. The sulfation pattern of HP is relatively equally distributed over the entire GAG chain. While in the biosynthesis of HS, the modification reactions occur in clusters along the GAG chain, resulting in an uneven sulfation pattern with distinct high-sulfation and low- or non-sulfation regions.

While HS PGs are produced in almost all mammalian cells and are widely distributed on the cell membrane and extracellularly, the HP PG is processed only in connective-tissue-type mast cells or basophils and is primarily localized in intracellular granules. HP has multi-faceted pharmacological functions, including its well-known anticoagulant activity, and other activities, including anti-inflammatory and anti-cancer activity (Fu et al. 2016; Köwitsch et al. 2018; Yip et al. 2006). The biological activity of HS is quite complex, with HS binding diverse ligands that are engaged in numerous biological processes such as angiogenesis, morphogenesis, ECM assembly, and regulation of the coagulation system. HS PGs have a significant role in developmental biology and cell signaling, and are widely engaged in various physiological and pathological processes, including embryonic development, adult tissue homeostasis, aging, and infection (De Pasquale and Pavone 2019; Kamhi et al. 2013).

4.2.2 Chondroitin Sulfate and Dermatan Sulfate

The CS/DS GAGs also occur as PGs and are *O*-glycosidically linked to the serine residues of their core proteins. The GAGs in this family contain repeating disaccharide units of →4) β-D-GlcA/α-L-IdoA (1 → 3) β-D-GalNAc (1→. The CS/DS family contains no *N*-sulfo group substitutions. CS GAGs are named according to their sulfation pattern, for example, CS-A for single sulfation at C4 of the GalNAc and CS-C for single sulfation at C6 of the GalNAc. The different CS GAGs occur in a species- and tissue-specific manner. The GlcA is the hexuronic acid found in CS, while DS consists of IdoA as the hexuronic acid (Volpi 2019). Cartilaginous tissues are a rich biological source of CS. DS, also known as CS-B, is an important component of the ECM of the connective tissues. CS/DS PGs are also widely distributed on cell surface and in the extracellular environment, carrying out diverse physiological functions especially those that are necessary in tissue development and repair, regulation of the central nervous system, and maturation of the organisms. CS is also well known as healthcare supplements that help relieve osteoarthritis (Pomin et al. 2019; Sugahara and Mikami 2007; Rauch and Kappler 2006).

4.2.3 Keratan Sulfate

KS are 6-*O*-sulfated GAGs with the disaccharide building block, →3) β-D-Gal(1 → 4) β-D-GlcNAc (1→. KS has a galactose residue in its backbone instead of uronic acid, making KS the only GAG lacking a carboxyl group. Sialic acids occasionally terminate KS chains. KS contains di-sulfated, mono-sulfated, and non-sulfated poly-*N*-acetyllactosamine regions and, thus, has

a non-uniform charge distribution. The KS PGs in human body mainly exist in the cornea, brain, and skeletal tissues, among which the cornea is the richest tissue source. It is subdivided into three classes according to the ways it is linked to the core protein. KS I (corneal type, as originally described in cornea) is *N*-glycosidically conjugated through a biantennary branched linkage region to an asparagine residue of its core protein. KS II (skeletal type, which is generally found in cartilage) is *O*-glycosidically linked through *N*-acetylgalactosamine (GalNAc) to a serine or threonine residue of its core protein. KS III is *O*-linked through mannose to serine, and is preferentially found in the PG of brain and nervous tissue (Köwitsch et al. 2018; Uchimura 2015). KS acting as a constitutive molecule of the extracellular matrices serves as a hydrating and a signaling agent in the cornea and cartilage tissues. KS is believed to be the newest GAG from an evolutionary perspective, and its biological functions are the least understood. Compared to the other classes of GAGs, KS has the least well-described interactive properties. An increasing number of studies have recently been undertaken demonstrating the interactive properties of KS with neuroregulatory proteins, growth factors, and cytokines, indicating varied roles for KS PGs in development and regenerative neural processes (Caterson and Melrose 2018; Melrose 2019; Funderburgh 2000).

4.2.4 Hyaluronan

HA has a simple repeating disaccharide unit of →4) β-D-GlcA(1 → 3)β-D-GlcNAc(1→. It is the only GAG that is neither sulfated nor linked to a protein core. Different from all other GAGs, HA in eukaryotic cells is biosynthesized at the inner surface of the plasma membrane catalyzed by membrane-bound hyaluronan synthases (HASs). Large HA aggregates are produced in cells and are extruded through the cell surface into the extracellular environment without further structural modification (Fig. 4.1a). HA non-covalently binds to a class of HA-binding proteins, called hyaladherins. In cartilage, HA interacts with hyaladherins, as well as a major cartilage proteoglycan-aggrecan (Hascall and Esko 2017). The binding of hyaladherins to HA assists aggrecans in locking on to an HA chain (Fig. 4.1b). HA solutions show viscoelastic properties due to the high molecular weight of HA. The physical properties of HA help keep the eyes humid and provide protection for the joints during motion. HA also exerts biological functions through interacting with its cell-surface receptors. It interacts primarily with two cell-surface proteins, CD44 and RHAMM. HA-receptor interactions engage in various biological and pathological events. They are involved in cellular adhesion, locomotion, proliferation, and signaling and individual development. They are also associated with inflammation and cancer progression. High levels of HA expression are normally correlated to a poor prognosis for cancer patients. The interactions of HA with its binding proteins are complicated due to the constant turnover and rapid metabolism of HA that produce hyaluronan molecules of different sizes with different bioactivities. Hyaluronan is a promising biomaterial used in wound healing or as scaffolds in surgery. HA has also been widely used for providing lubrication and mechanical support for the joints, or serving as a drug delivery material (Tamer 2013; Hascall and Esko 2017; Passi et al. 2019; Passi and Vigetti 2019).

4.3 Glycosaminoglycans and Diseases

GAGs are expressed in virtually all mammalian cells and widely participate in numerous biological processes. Some inherited disorders are caused by genetic defects in enzymes that are responsible for GAG metabolism. GAGs also play critical roles in the infectious process of pathogens. In addition, dysregulation of GAG expression is also observed in diseases such as cancers. Pathologically, the association of GAGs with various diseases is quite complicated; hence, we only make a brief introduction using a few cases in the following section.

4.3.1 Innate Disease

The impacts of genetic defects in enzymes associated with GAG metabolism have been demonstrated through in vitro and in vivo experiments, as well as in clinical cases. A large collection of mutant cells defective in GAG/proteoglycan biosynthesis has been isolated and cultured in vitro. Mutants in different GAG-biosynthesis enzymes fail to extend GAG chains, or produce GAG chains with a partial structural deficiency (Esko and Stanley 2017). The functions of GAGs in vivo have been revealed using model organisms such as nematodes, fruit flies, zebrafish, and knockout mice (Mizumoto et al. 2014). Mice deficient in enzymes responsible for GAG synthesis and modification often show embryonic or early postnatal lethality. In human beings, mutations in the genes encoding glycosyltransferases, sulfotransferases, and related enzymes responsible for the biosynthesis of GAGs cause a number of genetic disorders such as chondrodysplasia, spondyloepiphyseal dysplasia, Ehlers-Danlos syndromes, macular corneal dystrophy, and non-inflammatory peeling skin (Mizumoto et al. 2013; Freeze et al. 2017; Soares da Costa et al. 2017). Inborn errors in GAG metabolism, mucopolysaccharidoses (MPS), are caused by the deficiency of one of the many enzymes involved in GAG catabolism. As a group, MPS are relatively frequent, with an overall estimated incidence of around 1 in 20,000–25,000 births, although taken separately, each MPS disease is relatively rare. Current therapies are not curative but can improve life quality for many MPS diseases (De Pasquale and Pavone 2019; Sawamoto et al. 2019).

4.3.2 Infectious Disease

The extraordinary structural diversity of GAGs enables these biological macromolecules to interact with a wide variety of proteins and, therefore, modulate various biological processes. GAGs can also interact with pathogens (Aquino et al. 2010; Mycroft-West et al. 2018). Pathogens interact with host GAGs (mainly the HS/HP and CS/DS families) through their GAG-binding proteins to facilitate attachment and invasion. We now know that a wide variety of pathogens, including viruses, bacteria, parasites, and fungi, subvert GAGs in virtually all major steps of pathogenesis (Aquino and Park 2016). Infectious diseases have aroused serious concerns in recent years, including SARS-CoV (Kim et al. 2020), zika virus (Kim et al. 2017a, b), Ebola virus (Hearn et al. 2015), and spirochete bacterium *Borrelia burgdorferi*, which causes Lyme disease (Lin et al. 2017), and each involves a GAG–pathogen interaction.

There is some data showing that GAGs can function as direct internalization receptors for certain pathogens. However, GAGs are well known as initial attachment sites or co-receptors to facilitate the internalization of pathogens in cooperation with their primary entry receptors. For example, angiotensin-converting enzyme 2 (ACE2) is the primary receptor employed by the coronavirus for host cell entry. The research using coronavirus NL63 showed that directed expression or selective scission of the ACE2 had no measurable effect on virus adhesion and HS serves as an attachment receptor (Milewska et al. 2014). It was also recently found in the SARS-CoV-2 that the spike protein of the virus binds HS and the related GAG, HP, highlighting the potential of using HS/HP as therapeutic agents for treating SARS-CoV-2 infection (Kim et al. 2020; Liu et al. 2020; Mycroft-West et al. 2020; Partridge et al. 2020) .

Besides attachment and invasion, GAGs are also involved in pathogen transmission and dissemination. The interaction of HIV with HS on dendritic cells (DCs) has been shown to play a critical role in the transmission of HIV to T-cells. In addition, some pathogens use GAGs to evade host defense. For example, by secreting proteinases or virulence factors, pathogens can release GAG or PG fragments from host cells to bind to and neutralize positively charged antimicrobial peptides (AMPs). Some pathogens such as Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) can even produce HA GAGs, which is considered camouflage serving as protection from host defense attack (Aquino et al. 2010).

It is still a source of debate whether the interaction between pathogen surface proteins and

different GAGs are specific or promiscuous. However, some evidence suggests that there are specific interactions based on unique GAG structural features such as sulfation patterns and uronic acid epimers that promote the interaction between host GAGs and pathogen proteins (Kamhi et al. 2013; Liu et al. 2020). As we increase our understanding of the exact mechanisms of pathogen-GAG interactions, potential strategies might be discovered for using these interactions as a new frontier to combat infectious diseases.

4.3.3 Cancer

Cancer is a major disease across the world, which accounts for a large number of fatalities annually. GAGs and PGs both play significant roles in cancer-related processes, including cancer cell proliferation and growth, invasion and metastasis, angiogenesis, and the regulation of cancer-stem-cell functions (Yip et al. 2006; Vitale et al. 2019). Tumor cells usually have GAGs and PGs aberrantly expressed and displayed on their surface. This is the consequence of an altered expression of enzymes involved in GAG/PG biosynthesis and catabolism (Soares da Costa et al. 2017; Vitale et al. 2019; Afratis et al. 2012). GAGs in a tumor-cell environment also participate in cancer events by recruiting and contacting growth factors and interacting with tumor cell ligands (Yip et al. 2006). GAGs and related PGs, including HS/HP, CS/DS, and HA, have been widely studied in cancer research. The altered expression of HS and CS is a common feature of tumor cells and is known to play a key role in malignant transformation and tumor metastasis (Rangappa and Sugahara 2014). HA has been extensively studied as a principal constituent of tumor stroma. HA interacts with CD44, the main HA receptor on the tumor cell surface. This interaction is important in cancer progression (Morla 2019; Vitale et al. 2019; Passi et al. 2019).

The altered expression of GAGs/PGs in tumor events can be both quantitative and structural. For example, a decrease in the levels of HS is found in some cancers, resulting in the malignant cells becoming more invasive (Morla 2019). Changes in the degree and pattern of GAG sulfation, for example, an altered 6-*O*-sulfation, are also commonly reported in cancers (Soares da Costa et al. 2017). It is noteworthy that GAGs have complicated and variable roles in cancer events. Different kinds of GAGs have different impacts on cancer progression, which can be either facilitative or inhibitive. Even the same kind of GAGs and PGs, for example, HSPGs, may act as either inhibitors or promoters of tumor progression, depending on the type and stage of the disease. In addition, a change in the mode of GAG expression, either quantitatively or structurally, is also variable in different tumor types, as well as at different stages of the same cancers (Morla 2019; Soares da Costa et al. 2017). In addition, person-to-person variations in GAG composition should also not to be neglected. GAGs are increasingly being considered potential cancer biomarkers, the innate person-to-person variations have to be taken into account as significant factors in developing precision medicines (Soares da Costa et al. 2017; Weyers et al. 2012).

In addition to the above-mentioned aspects, GAGs and PGs are also associated with other pathological processes, such as binding amyloid β proteins (Abs) and developing Alzheimer's disease (AD) (Ariga et al. 2010), or engaging in inflammation and autoimmune diseases (Morla 2019). GAGs and PGs are involved in the pathophysiology of almost every major disease. The increasing understanding of their roles in these diseases will help us find better solutions for treatment.

4.4 "Sweet" Therapeutics

Carbohydrates serve as building units of biological molecules. Our cells run on them. So, it is not a surprise to find carbohydrates and glycosylated molecules applied in treating various diseases (Paderi et al. 2018). Small molecular chemicals such as monosaccharides and their analogs have proven potential in medicine development, and have already been applied clinically (Miyoshi et al. 2020). For example, administration of the mannose monosaccharide suppresses tumor growth in vitro and in mouse models (Gonzalez

et al. 2018). The monomeric unit of chitin, *N*-acetylglucosamine (GlcNAc), a major component in GAG chains, has been widely applied as a dietary supplement. The sialic acid analogs Zanamivir and Oseltamivir have been used clinically for years as antiviral medications against influenza. Glycosylation can improve traditional chemodrugs by allowing their selective targeting to tumor cells, and reducing the drug remains in blood circulation, as well as toxicity towards normal cells (Molejon et al. 2020). Compared to the small molecular compounds, the glycosylated biotherapeutics have become more appealing in recent years. In the market for glycosylated biotherapeutics, glycoproteins are the most widespread agents, and monoclonal antibodies (mAbs) are the most commonly prescribed (Madsen et al. 2018). Since GAGs are involved in a plethora of biological activities and pathogenesis, their application in medicine development has been of long interest in the pharmaceutical industry. Many GAG-based drugs are being developed with encouraging results in animal models and clinical trials, revealing their potential for development as therapeutics.

4.4.1 Application of Glycosaminoglycans and Mimetics

The best-studied carbohydrate therapeutic to date is heparin, the first GAG-based drug used as an anticoagulant. CS has been recommended for use in relieving osteoarthritis. KS is also used for the treatment of corneal dystrophy (Soares da Costa et al. 2017). Considering the diverse biological functions of GAGs and their universal involvement in pathological processes, it is reasonable to infer that they have a vast potential for treating different diseases. As expected, there have been numerous articles confirming the biological activities of GAGs. In addition to the well-known impact of anticoagulants and in relieving osteoarthritis, GAGs are highlighted for their potential as therapeutics for treating tumor, inflammation, infectious diseases, and neurological diseases (Köwitsch et al. 2018; Morla 2019; Belting 2014; Gulati and Poluri 2016; Mycroft-West et al. 2018; Pomin et al. 2019; Hayes and Melrose 2018). In addition, GAGs have been used as biomaterials in a variety of biomedical applications, primarily for wound healing, drug delivery, and tissue engineering purposes (Ghatak et al. 2015; Köwitsch et al. 2018). HA is quite attractive among GAG family members for use as a biomaterial, since it can already be produced by biotechnological approaches, in high purity and with well-controlled composition (Passi and Vigetti 2019).

Although GAGs have shown remarkable bioactivities, there are few current clinical applications of natural sulfated GAGs. Isolating GAGs from natural sources is costly and labor intensive, and significant batch-to-batch variation makes quality control a daunting task. Therapeutic GAGs also have a concern over impurities and contamination in their extraction from mammalian tissues, although this purification approach is currently the most common source for therapeutic GAGs. Using synthetic methods (chemical and/or enzymatic) to prepare GAGs is a more reliable strategy to obtain therapeutic GAGs and avoid their inherent heterogeneity. Another worry when applying GAGs as therapeutics is that they are polypharmacological agents that play essential roles in many endogenous processes and, thus, may cause some side-effects due to their compromised selectivity. For example, heparin, in addition to its anticoagulant properties, also possesses anti-cancer activities. However, its anticoagulant property limits its application in treating cancer (Morla 2019). GAG mimetics can serve as a solution for this issue. GAG mimetics can be classified into two classes: saccharide-based and non-saccharide-based. They are both typically synthetic. Saccharide-based GAG mimetics are built on a sugar backbone, while the non-saccharide-based mimetics use non-sugar-based scaffolds. Some of these mimetics are completely homogenous molecules carrying negative charges through sulfates, sulfonates, carboxylates, and/or phosphates. Both kinds of GAG mimetics have been developed for the treatment of cancer and inflammation, and some of these are currently in clinical trials or in clinical use (Morla 2019). There are also some unique GAG-like carbohydrates discovered from invertebrate animals, especially marine invertebrates (Pomin

2015; Mycroft-West et al. 2018; Vasconcelos and Pomin 2017). Their bioactivity and potential as therapeutics are being studied.

4.4.2 Therapeutics Based on the Role of GAGs in Pathogenesis

GAGs can serve as therapeutics but they also play a less direct role in pathophysiology and can be inspirations for new drug development. As GAGs are involved in key steps of pathogenesis, GAG-pathogen interactions are potential targets for the development of both highly specific and broadly effective antimicrobial/antiviral therapies (Lin et al. 2017; Kim et al. 2017a). Exogenous GAGs or GAG mimetics that bind the pathogen proteins, and the cationic compounds that bind the endogenous GAGs can both serve as obstructions in the pathogen's interaction with endogenous GAGs and, therefore, can hamper pathogen invasion. There are several engineered GAGs, sulfated compounds, and cationic compounds that show potentials in treating infectious diseases (Aquino et al. 2010). However, caution must be taken in applying GAGs as therapeutics for treating infection since they have wide context-dependent functions that may cause undesirable side-effects.

As mentioned previously, GAGs are considered biomarkers in a variety of malignant tumors. Therefore, GAGs and PGs can be used as tumor-specific targets for drug delivery (Afratis et al. 2012). Liposomes are used for selective drug delivery in certain metastatic tumors due to their over-expression of CS. Monoclonal antibodies and other protein/peptide binding to specific GAGs and PGs can also be used for targeting tumor cells. For example, a parasite-derived recombinant protein was used to specifically bind placental CS chains that were expressed in malignant cells. Conjugation with diphtheria toxin or to hemiasterlin compounds, this malaria protein can strongly inhibit in vivo tumor cell growth and metastasis (Salanti et al. 2015). GAGs can also be used as tumor-targeting vehicles for drugs. HA, as an ECM constituent interacting with its CD44 receptor on the tumor cell surface, has been employed for delivering drugs to tumor cells. It can be used as a drug carrier and ligand on liposomes or nanoparticles targeting tumor cells that over-express CD44 (Passi and Vigetti 2019). Another strategy is targeting the GAG-related enzymes. HS mimetics, which can serve as inhibitors of HS-degrading enzymes, are widely studied (Nurcombe et al. 2019). There are other strategies such as using growth factor (GF)-binding molecules to interfere with GAG-GF interactions (Belting 2014). A better understanding of the molecular mechanisms by which PGs and GAGs engage in cancer progression could facilitate the development of targeted therapeutic approaches, and eventually benifit the patients.

4.5 From Past to the Future

The advancement of research on carbohydrates has been driven by a number of outstanding chemists beginning in the nineteenth century (Varki and Kornfeld 2017b). Stereoisomeric structures of monosaccharides were discovered in 1890s (Hudson 1941). The most famous member of GAG family, heparin, was first reported and named in 1916 despite its still unrevealed composition. From 1922 onwards, partially purified heparin became commercially available (Mutt and Blombäck 2000). When heparin had become a research hotspot in both academic and industrial laboratories in the first part of the twentieth century, the other members of GAG family also gradually came to be known. CS and HA were respectively characterized in 1925 and 1934. Eric Jorpes identified heparan sulfate in 1948 (Varki and Kornfeld 2017b). In the subsequent decades, due to the development of separation and analytical techniques, the biological functions of carbohydrates in living organisms were revealed. It became increasingly evident that carbohydrates serve as far more than energy sources and structural materials; instead they widely engaged in biological and pathological processes such as cellular recognition and migration, virus infection, and ontogenesis. In late 1980s, the word "glycobiology" was first coined, marking a new frontier pursuing a modern understanding of the cell and molecular biology of gly-

cans. Glycobiology has now become one of the most rapidly growing fields in the natural sciences.

When it comes to the "-omics" era for glycans, this feature is highlighted by using a systems-level analysis to describe many aspects of glycobiology (Rudd et al. 2017). The revolutionary invention of structural analysis technology in the twentieth century, especially the mass spectrometry based on soft ionization (ESI, MALDI), has remarkably accelerated the research on carbohydrates. And during the past two decades, benefiting from advanced separation techniques, the state-of-the-art mass spectrometry, and high-throughput microarray technologies, the capacity of analyzing glycan complexes has continuously improved. This has ensured a position for glycomics alongside other mainstream omics fields (genomics, proteomics, etc.). Although they share a common "ome" postfix, the glycome differs from its counterparts, the genome and proteome, as glycans are not produced in template-driven synthesis, as are DNA and proteins. However, the studies of glycosylation-related genes and glycan-protein interactions can serve as bridges connecting glycans with their counterpart macromolecules, DNAs and proteins, and allow us an increased understanding of the critical meanings of glycans in biological processes.

Information from glycan-ligand interactions is critical in understanding the biological roles of glycans. GAGs carry out numerous physiological functions by their interaction with protein ligands. Their interactions are often specific, requiring a defined GAG sequence or domains. Therefore, detailed structural information of GAG chains is required for exploring the mechanisms of GAG-ligand interactions. Compared to other members in human glycome, GAGs have more complicated structures. The development of GAG sequencing has lagged the sequencing of their macromolecular counterparts, proteins and DNA, resulting in a bottleneck in the structural elucidation of GAG chains. Thus far, there have been only two cases of reported GAG sequencing, relatively simple GAGs, bikunin (Ly et al. 2011) and decorin (Yu et al. 2017). However, a variety of advanced techniques have emerged over the past two decades, remarkably enhancing GAG sequencing. State-of-the-art MS techniques, such as high-resolution mass spectrometry and tandem MS, have been major technical achievements responsible for much of the success in GAG analysis. A variety of separation techniques adapted to MS coupling, including size-exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC), reverse phase ion-pairing LC (RPIP-LC), porous graphitized carbon (PGC), capillary electrophoresis (CE), and the emerging ion mobility (IM) method, have significantly facilitated the application of MS in GAG analysis. The glycan microarray represents another major development in glycoscience (Turnbull and Field 2007; Rillahan and Paulson 2011; Smith et al. 2019). This uses omics to reveal the functional roles of glycans, by affording a high-throughput interrogation of interactions between diverse glycans and their glycan-binding protein (GBP) partners. The developments of recombinant GAG-degrading enzymes (Sasisekharan et al. 2006), GAG synthesis techniques (Pomin and Wang 2018), and bioinformatics tools, have also made great contributions to GAG research. Sensor technologies like surface plasmon resonance (SPR) have served as a powerful tool in illustrating GAGs' function.

Glycoscience is a rising research field. Glycome research can help facilitate the development of personalized medicine, which is a promising solution for the remarkable inter-individual differences in glycosylation that cause different responses to standard therapies (Weyers et al. 2012). Glycome research can also provide advanced solutions to treat diseases such as virus infections, allowing efficient reaction in public health emergencies such as a coronavirus pandemic, facilitating development of drugs and vaccines. Glycome research also deepens our knowledge in the field of genetics. Glycosylation is a non-template-driven process that can be affected by both genetic and environment factors, resulting in variable glycosylated biomolecules. These glycosylated products enable various biological effects, even including the alteration of gene expression. The

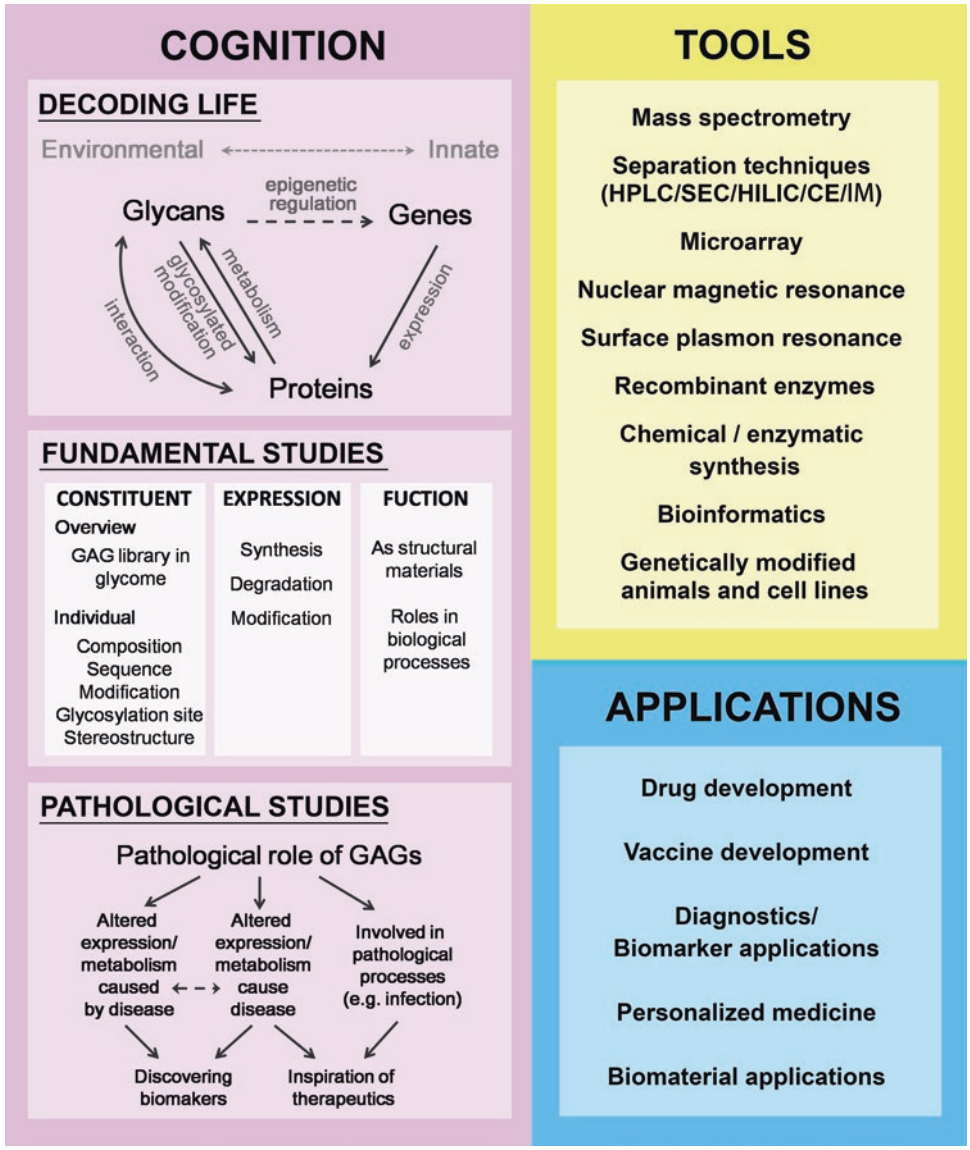

Fig. 4.2 The contents of glycosaminoglycans, in the categories of recognition, tools, and applications, are explored

regulation of and by glycosylation is quite a challenging topic in epigenetics research (Kizuka 2021). In spite of its vast space for future exploration, glycomics studies are still in their preliminary stages compared to genomics and proteomics studies. Glycans are considered the "dark matter" of the biological universe (Varki and Kornfeld 2017a). Despite their noticeable existence, our understanding of these biomolecules, such as their roles in complex biological systems, how they are influenced by innate and environmental factors, and their correlations with other biological molecules, is still quite limited. GAGs are especially complicated as a glycome constituent. But still we believe by the virtue of future development of cutting-edge technologies, the exploration on GAGs (Fig. 4.2) can parallelly progress on other glycome members. This increased knowledge will keep shedding new light on this biological "dark matter," and eventually lead us to the future when the glycobiology finally fully merges into a holistic approach to biological systems.

Compliance with Ethical Standards

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Other Types of Glycosylation

5

Yohei Tsukamoto and Hideyuki Takeuchi

Abstract

O-Linked glycosylation such as *O*-fucose, *O*-glucose, and *O*-*N*-acetylglucosamine are considered to be unusual. As suggested by the high levels of evolutional conservation, these *O*-glycans are fundamentally important for life. In the last two decades, our understanding of the importance of these glycans has greatly advanced. In particular, identification of the glycosyltransferases responsible for the biosynthesis of these glycans has accelerated basic research on the functional significance and molecular mechanisms by which these *O*-glycans regulate protein functions as well as clinical research on human diseases due to changes in these types of *O*-glycosylation. Notably, Notch receptor signaling is modified with and regulated by these types of *O*-glycans. Here, we summarize the current view of the structures and the significance of these *O*-glycans mainly in the context of Notch signaling regulation and human diseases.

Keywords

O-glycans · *O*-fucose · *O*-glucose · *O*-GlcNAc · EGF · Notch

5.1 Introduction

O-Linked glycosylation is a relatively newly defined class of glycosylation. In particular, *O*-fucose, *O*-glucose, and *O*-*N*-acetylglucosamine (GlcNAc) glycans, which modify numerous membrane proteins such as Notch receptors and secretory proteins, have been focused on in this chapter. Previous studies have revealed the specific amino acid consensus sequences are required for the attachment of these glycans. These glycans have important biological functions and are unique in that the molecular weight of these glycans is relatively small. Further, even if they are extended, they only form a trisaccharide or tetrasaccharide structure. However, not much is known about these glycans because tools such as antibodies or lectins for research related to glycans are very limited. In recent years, improvements in analytical techniques, methods, and instruments have enabled us to understand many

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things about these glycans. There are not many proteins that have been confirmed to have been modified by these glycans. Nonetheless, existing information suggests that these glycans play important biological roles.

Notch receptors are outstanding in that they are heavily modified with and regulated by these *O*-linked glycans (Urata and Takeuchi 2020). The Notch receptor-mediated intercellular signaling pathway is important for the development and homeostasis of multicellular organisms (Kopan and Ilagan 2009). Dysregulation of Notch signaling leads to various human diseases (Siebel and Lendahl 2017). Genetic and biochemical studies have revealed that *O*-linked glycosylation on Notch receptors is essential for the transduction of Notch signaling. Mammals have four *NOTCH* paralogs whereas *Drosophila* has a single *Notch* gene. Notch receptors are type I membrane protein receptors with 29-36 epidermal growth factor-like (EGF) repeats in the extracellular domain. There are two different classes of Notch ligands, *Delta* and *Serrate*, in *Drosophila*. Mammals have two activating *Delta*-like ligands (DLL) DLL1 and DLL4 and have two activating *Serrate*-like ligands, JAGGED1 and JAGGED2. Notch ligands are also a Type-I transmembrane protein which contains EGF repeats. *O*-fucose, *O*-glucose, and *O*-GlcNAc glycans are added to EGF repeats. Although both Notch receptors and ligands are modified with these *O*-glycans, the biological functions of Notch receptors are clearly regulated by these *O*-glycans.

5.2 *O*-Fucose Glycans

5.2.1 *O*-Fucose Glycans on EGF Repeats

O-Fucose glycans found on EGF repeats are known to be extended by GlcNAc, galactose, and *N*-acetylneuraminic acid (Neu5Ac) in mammals (Fig. 5.1) (Moloney et al. 2000). The most mature *O*-fucose glycan forms a tetrasaccharide structure, Neu5Acα2-3/6Galβ1-4GlcNAcβ1-3Fuc-*O*-Ser. In the endoplasmic reticulum (ER), protein *O*-fucosyltransferase 1 (POFUT1) transfers *O*-fucose from GDP-fucose to a serine or threonine residue in the consensus motif, $C^2XXXX(\underline{S/T})C^3$, of EGF repeats (the superscript above C indicates the number of conserved cysteines of the EGF repeat counted from the N-terminal, X indicates an arbitrary amino acid, and underlined residues are *O*-fucosylation sites) (Holdener and Haltiwanger 2019). POFUT1 is composed of two subunits that interact with a sugar donor substrate GDP-fucose by burying it in the gap in between (McMillan et al. 2017). GlcNAc can be attached to *O*-fucose transferred by POFUT1. GlcNAc extension is catalyzed by β-1-3-*N*-acetylglucosaminyltransferase, also called Fringe. Mammals have three paralogs of Fringe, viz., Lunatic Fringe (LFNG), Manic Fringe (MFNG), and Radical Fringe (RFNG), and they are localized in the Golgi apparatus (Johnston et al. 1997). Furthermore, galactose and sialic acid are added to *O*-fucose glycans by galactosyltransferase(s) and sialyltransferase(s) in the Golgi apparatus (Moloney et al. 2000). There are nearly 100 proteins with the consensus sequence for POFUT1-mediated *O*-fucose glycosylation (Rampal et al. 2007; Schneider et al. 2017). The importance of *O*-fucose glycans has been well studied in terms of *O*-fucose and its GlcNAc elongation by Fringe. It is known that loss of *O*-fucose glycans due to the loss of *POFUT1* in human embryonic kidney cells (HEK293T cells) results in the impaired trafficking of endogenous NOTCH1 proteins to the cell surface, and the genetic deletion of *Pofut1* markedly reduced Notch signaling, which leads to embryonic lethality (Takeuchi et al. 2017; Taylor et al. 2014; Luca et al. 2015, 2017). The *O*-fucose glycans positively regulate the stability of EGF repeats of the Notch receptor. Thus, the loss of the *O*-fucose glycans results in the lack of stability of the EGF repeats. It is proposed that unstable proteins are recognized as abnormal and are sent to the degradation pathway (Fig. 5.2) (Takeuchi et al. 2017). *O*-Fucose glycans differentially regulate the interactions between Notch receptors and two classes of Notch ligands. The binding of *O*-fucose glycan-lacking NOTCH1 receptors to Notch ligands DLL1 and DLL4 is reduced in comparison to Notch with *O*-fucose

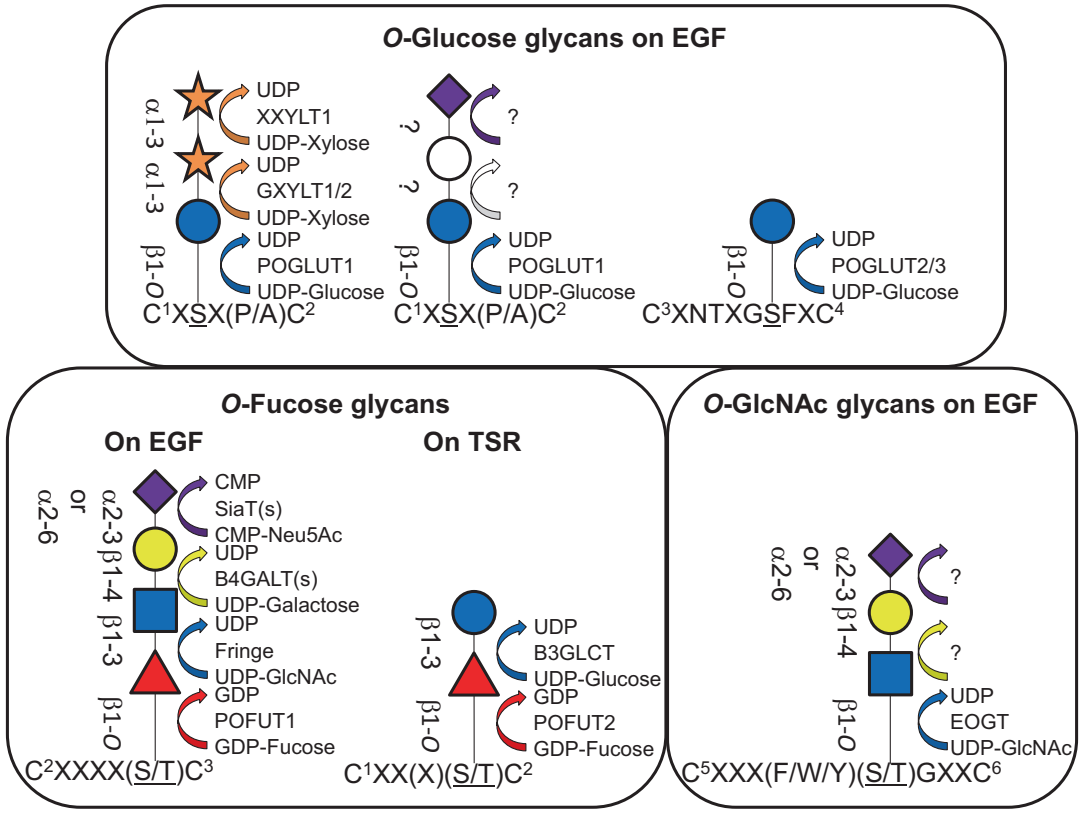

Fig. 5.1 Schematics of "Other types of glycans" described in this chapter
The amino acid consensus sequences for each glycosylation in epidermal growth factor-like (EGF) repeats and thrombospondin type-I repeats (TSR) are indicated. The glycosyltransferases responsible are also indicated. Blue circle: glucose; orange star: xylose; white circle: hexose; purple diamond: *N*-acetylneuraminic acid (Neu5Ac); yellow circle: galactose; red triangle: fucose: blue square: *N*-acetylglucosamine (GlcNAc)

glycan (Kakuda and Haltiwanger 2017; Luca et al. 2015). *O*-Fucose glycans are located at the position where they contact the ligand directly. Thus, *O*-fucose glycans directly regulate the binding ability of NOTCH1 to its ligands (Kakuda and Haltiwanger 2017). It has also been reported that *O*-fucose glycosylation and Fringe elongation of *O*-fucose glycans are important for binding of NOTCH1 to JAGGED1, not only in the region directly interacting with the ligand but also in other regions (Kakuda and Haltiwanger 2017). Cell-based Notch signaling reporter assays combined with mass spectrometric analysis showed that the elongation of *O*-fucose glycans by all three types of Fringe promoted NOTCH1 activation by DLL1, whereas that by LFNG and MFNG inhibited JAG1-mediated NOTCH1 activation (Fig. 5.3) (Kakuda and Haltiwanger 2017). Specifically, the elongation of *O*-fucose glycans at EGF8 and EGF12 in NOTCH1 is necessary for the promoting effect of the Fringes on DLL1-mediated NOTCH1 activation. The elongation of *O*-fucose glycans at EGF6 and EGF36 in NOTCH1 is necessary for the inhibitory effect of the Fringes on JAG1-mediated NOTCH1 activation (Kakuda and Haltiwanger 2017). A recent study from the same group showed that, as for NOTCH2, LFNG enhances the activation by DLL1, and MFNG suppresses the activation by JAGGED1 and JAGGED2 (Fig. 5.3) (Kakuda et al. 2020). In addition to the Notch receptors, *O*-fucose modification of Wnt

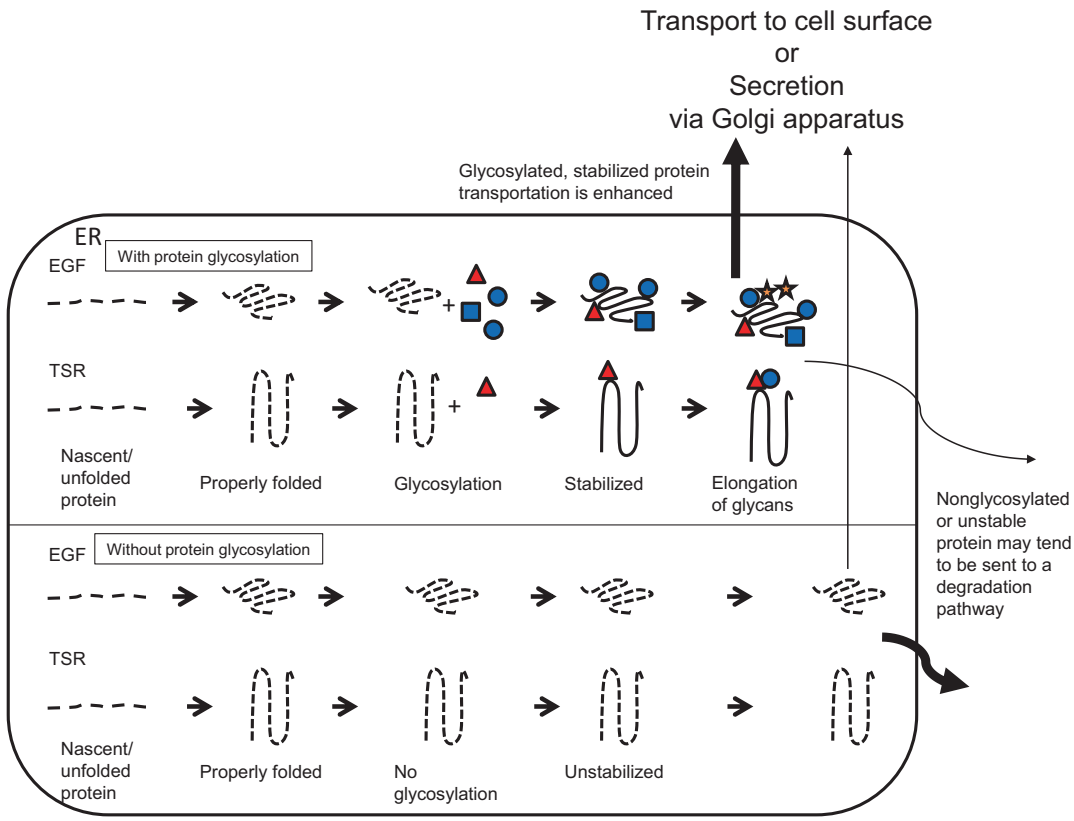

Fig. 5.2 Potential roles of *O*-glycans in quality control of EGF repeat- or TSR-containing proteins
When epidermal growth factor-like (EGF) repeats and thrombospondin type-I repeats (TSRs) are folded in the endoplasmic reticulum (ER), *O*-glycans such as *O*-glucose (Takeuchi et al. 2012, 2017), *O*-fucose (Takeuchi et al. 2017; Vasudevan et al. 2015), and *O*-*N*-acetylglucosamine (*O*-GlcNAc) (Sawaguchi et al. 2017; Sakaidani et al. 2012) are transferred only to the properly folded EGF repeats and TSRs. The *O*-glycans make EGF repeats and TSRs more stable, possibly through intramolecular interactions between *O*-glycans and backbone polypeptides, which results in the enhancement of their trafficking to the cell surface and secretion. Those that have not been modified are unstable and probably go to the degradation pathway. Blue circle: glucose; orange star: xylose; red triangle: fucose; blue square: *N*-acetylglucosamine (GlcNAc): dashed line: unstable protein; solid line: stabilized protein

inhibitory factor 1 (Wif1) and Agrin was also observed. When a mutation of the *O*-fucose modification site of Wif1 is introduced, the secretion of Wif1 is reduced to less than half of that of wild-type Wif1 (Pennarubia et al. 2020b). As for Agrin, the loss of *O*-fucose modification promotes acetylcholine receptor clustering and MuSK phosphorylation in myotubes in culture. *POFUT1* deficiency also increases acetylcholine receptor aggregation in myotubes in adult skeletal muscles (Kim et al. 2008; Karlsson and McGuckin 2012). In the *Pofut1*-deficient mice, some phenotypes were observed similar to those in RBPJκ-deficient mice, such as developmental defects in mesodermal somites, neural tubes, blood vessels, and the heart (Okamura and Saga 2008b). In particular, valve formation in the heart has been reported to be severely affected, charac-

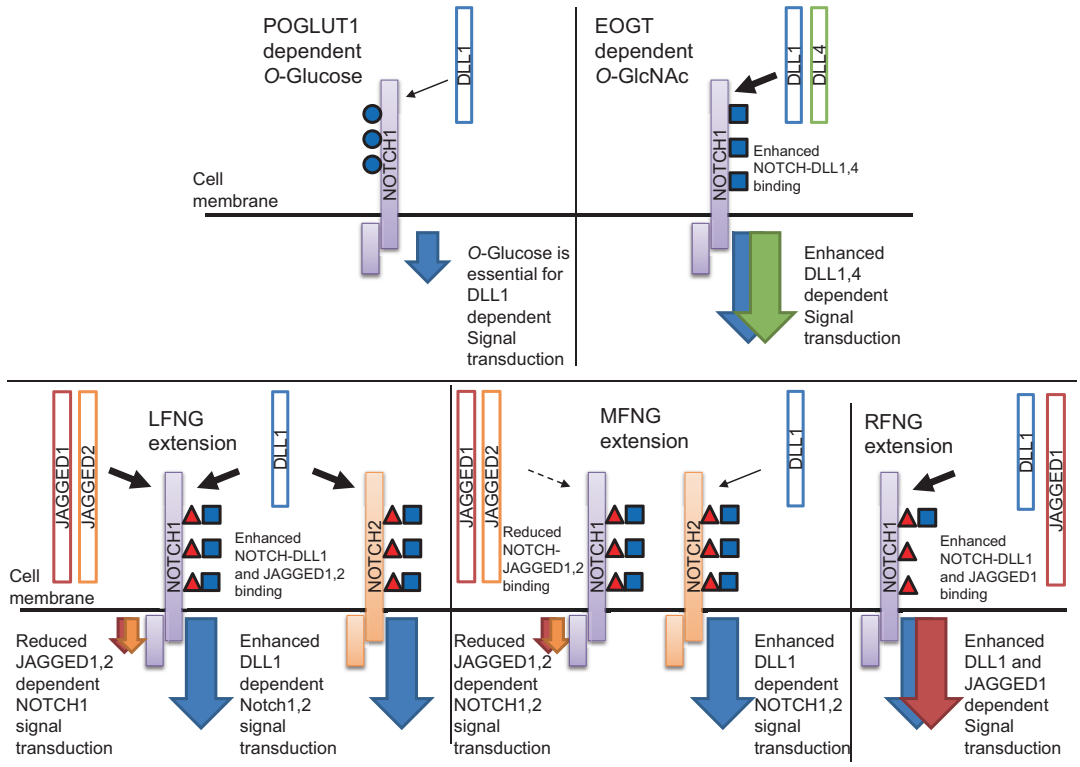

Fig. 5.3 Effects of *O*-glycans on Notch ligand binding and signal transduction
O-Fucose, *O*-glucose, and *O*-*N*-acetylglucosamine (GlcNAc) glycans in Notch receptors are important for ligand binding and signal transduction. *O*-Glucose glycans modified by POGLUT1 are essential for DLL1-dependent NOTCH1 signal transduction (*top left*) (Rana et al. 2011). *O*-GlcNAc glycan modified by EOGT enhances NOTCH-DLL1 and 4 binding, and DLL1- and DLL4-dependent signal transduction (*top right*) (Sawaguchi et al. 2017). Lunatic Fringe (LFNG)-elongated *O*-fucose glycans enhance NOTCH1-DLL1, JAGGED1, and JAGGED2 binding, and DLL1-dependent NOTCH1 signal transduction, NOTCH2-DLL1 binding, and DLL1-dependent NOTCH2 signal transduction, and reduce JAGGED1 and JAGGED2-dependent NOTCH1 signal transduction (*bottom left*). The Manic Fringe (MFNG)-elongated *O*-fucose glycans enhance NOTCH1-DLL1- and DLL1-dependent NOTCH1 signal transduction, DLL1-dependent NOTCH2 signal transduction, and reduce JAGGED1 and JAGGED2 binding and JAGGED1- and JAGGED2-dependent NOTCH1 and NOTCH2 signal transduction (*bottom middle*). Radical Fringe (RFNG)-elongated *O*-fucose glycans enhance NOTCH1-DLL1 and JAGGED1 binding, DLL1- and JAGGED1-dependent NOTCH1 signal transduction (*bottom right*) (Kakuda and Haltiwanger 2017; Kakuda et al. 2020). Solid arrow: NOTCH-ligand binding; dashed arrow: reduced NOTCH-ligand binding; bold and solid arrow: enhanced NOTCH-ligand binding

terized by the lack of mesenchymal cells seen in RBPJκ-deficient mice. Ultimately, embryonic development in *Pofut1*-deficient mice is arrested. Defects in valve formation due to the lack of epithelial-mesenchymal transition and defects in trabeculae were also observed in cardiac-specific *Pofut1*-deficient mice (Okamura and Saga 2008b). These phenotypes were partially rescued

by the forced overexpression of the Notch intracellular domain, suggesting that *POFUT1* deficiency affects Notch signaling but also affects other molecular pathways (Okamura and Saga 2008b). Mice deficient in *Pofut1* in the heart cause excessive angiogenic cell proliferation and neuroplex formation, leading to abnormal coronary arteries, myocardial infarction, and heart failure (Wang et al. 2017). These defects significantly affect cardiac function and cause severe myocardial hypoxia, leading to heart failure, rupture of coronary artery aneurysms, and early death in mice. The intracellular domains of the Notch ligands DLL4 and NOTCH1 are reduced in the endocardium and coronary artery endothelium of these mice (Wang et al. 2017). This suggests that these phenotypes are affected by reduced Notch signaling (Wang et al. 2017). In addition, it has been reported that mice lacking *Pofut1* in neural crest cells have impaired development of the enteric nervous system and die within 1 day of birth (Okamura and Saga 2008a). It has also been found that the expression of Hes1, a target gene of Notch1, was downregulated in the intestinal tract of these mice, suggesting that abnormalities in Notch signaling may be responsible for this defect (Okamura and Saga 2008a).

Some human disorders have been reported to result from a deficiency of *O*-fucose glycans on EGF repeats. Dowling-Degos disease (DDD) is a flaccid condition characterized by hyperpigmentation of the trunk and extremities, with pain and pruritus. Deficiency of *O*-fucose and *O*-glucose glycans is likely to be associated with DDD since mutations of *POFUT1* and *POGLUT1* encoding protein *O*-glucosyltransferase 1 (discussed later) are often discovered in patients with DDD. Most of the mutations discovered in *POFUT1* are nonsense or frameshift mutations, which result in the biosynthesis of truncated POFUT1 proteins (Kiuru et al. 2020; Atzmony et al. 2019; Kono et al. 2015, 2019; Li et al. 2013, 2016; Buket Basmanav et al. 2015). Truncated POFUT1 proteins do not form two subunits properly, possibly leading to serious defects in substrate recognition and glycosyltransferase activity. In addition, missense mutations in *POFUT1* have also been reported (McMillan et al. 2017; Buket Basmanav et al. 2015). Most of them are thought to cause abnormalities in the conformation and charge of the two subunits of POFUT1 proteins, resulting in the loss of enzymatic activity. In patients with these mutations, melanocytes, which produce melanin, and keratinocytes, which form the epidermis, Notch signaling is reduced (Atzmony et al. 2019). These Notch defects appear to result in abnormal pigmentation and epidermal keratinization.

Overexpression of *POFUT1* has been reported in hepatocellular carcinoma (Ma et al. 2016). It was found that Caveolin-1 (Cav-1) promotes the phosphorylation of HNF4A, Sp1, c-Myc, and CREB, which bind to the *POFUT1* promoter region, and that Cav-1 activates MAPK, which promotes *POFUT1* transcription. Notch signaling was activated in hepatocellular carcinoma cells, suggesting that upregulation of *POFUT1* enhanced the *O*-fucose glycosylation of Notch by POFUT1, which promoted Notch signaling. In vitro experiments in *POFUT1*-overexpressing hepatocellular carcinoma cells showed that cell proliferation and migration were promoted (Zhang et al. 2019a). Furthermore, the binding of these *POFUT1*-overexpressing cells to DLL1 is enhanced compared to that of parental cells (Ma et al. 2016). The knockdown of *POFUT1* in hepatocellular carcinoma cells significantly suppressed Notch1, cyclin D1, and p53, resulting in cell cycle arrest, proliferation, and adhesion inhibition. This suggests that enhanced *O*-fucose modification of Notch by POFUT1 in hepatocellular carcinoma affects the malignant traits of cancer (Annani-Akollor et al. 2014). Upregulation of *POFUT1* gene expression has also been reported in colorectal (Deschuyter et al. 2020; Lv et al. 2019; Komor et al. 2020), breast (Wan et al. 2017), stomach (Dong et al. 2017), oral cavity (Yokota et al. 2013), lung (Leng et al. 2018), and esophageal cancers (Sadeghzadeh et al. 2020). Nonsynonymous substitutions in *POFUT1* have also been found in colorectal cancer. This mutation is reported to enhance the enzymatic activity of POFUT1 (Deschuyter et al. 2020), suggesting that the enhanced *O*-fucosylation may contribute to the

increased malignancy in colorectal cancer (Deschuyter et al. 2020).

Aberrant expression of *POFUT1* has also been linked to other diseases in addition to cancer. Mutations in *POFUT1* have been found in patients with microcephaly (Takeuchi et al. 2018b). These mutations significantly reduce the enzymatic activity of POFUT1 and reduce Notch signaling, suggesting that reduction of *O*-fucose glycans on Notch is associated with the pathogenesis of microcephaly. POFUT1 expression is significantly reduced in the endometrium in patients with miscarriage (Liu et al. 2014). POFUT1 promotes the outgrowth, migration, and invasion of trophoblasts. These are important for trophoblast implantation. This is thought to regulate accurate development through the following mechanisms. POFUT1 expression is promoted during proper embryo implantation by progesterone, and epiregulin activates c-Fos and c-Jun (Cui et al. 2019, 2020). POFUT1 is highly expressed in trophoblasts of the embryo and endometrial stromal cells of the uterus. Urokinase-type plasminogen activator (uPA) is modified with *O*-fucose by POFUT1 (Zhang et al. 2019b). uPA *O*-fucose modification enhances PI3K/Akt signaling (Liu et al. 2017). This may promote proliferation, invasion, and adhesion of trophoblast cells. POFUT1 is also important for angiogenesis and vascular remodeling in endometrial stromal cells (Zhang et al. 2019b). This angiogenesis and defective remodeling are important for the formation of decidua from endometrial stromal cells.

Certain human diseases are associated with Fringe-dependent elongation of *O*-fucose glycans. A patient was found to have a mutation in *LFNG* in congenital scoliosis (Takeda et al. 2018; Otomo et al. 2019). The disease is caused by spinal malformation, and the main symptom is the lateral curvature of the spine. The mutation was a missense or frameshift mutation. In vitro GlcNAc-transferase activity assay showed that the activity of the LFNG with the mutation was substantially reduced compared to that of wild-type LFNG. This suggests that one of the causes of congenital scoliosis may be a defect of the Fringe elongation on *O*-fucose glycans.

Loss of *LFNG* induces basal-like breast cancer (Xu et al. 2012). Low expression of *LFNG* in humans is associated with high-grade tumors, triple-negative ERα/PR/HER2 status, and basal-like molecular subtypes (Xu et al. 2012). LFNG inhibits JAGGED-dependent Notch receptor activation, and promotes DLL-dependent Notch receptor activation via elongation of *O*-fucose glycan on Notch (Xu et al. 2012). Loss of *Lfng* results in increased JAGGED1-dependent activation of Notch, which upregulates c-Myc, CyclinD1, IgF1R, and uPA, and promotes cell proliferation in breast cancer mouse model. Furthermore, another article reported that loss of *Lfng* inhibits DLL1-dependent Notch signaling in bipotent mammary progenitor cells and causes basal cell proliferation (Bouras et al. 2008). Downregulation of Notch-targeted genes, such as HES/HEY, may negate the repression of the MET promoter. Loss of *Lfng* induces mammary tumorigenesis, and gene amplification of Met/Caveolin increases MET and CAVEOLIN1/2 protein expression. This increase in protein expression enhances Igf-1R and IRS-mediated signaling. These may result in breast cancer (Xu et al. 2012).

5.2.2 *O*-Fucose Glycans on Thrombospondin Type-I Repeats (TSRs)

In addition to EGF repeats, a similar protein domain called TSR is also modified by *O*-fucose glycans; unlike EGF repeats, this *O*-fucosylation is mediated by POFUT2 (Fig. 5.1). POFUT2 transfers *O*-fucose to a serine or threonine residue of $C^1XX(X)(\underline{S/T})C^2$ in a properly folded TSR in the ER (Martinez-Duncker et al. 2003; Schneider et al. 2017). Subsequently, the *O*-fucose is elongated to a disaccharide structure, Glcβ1-3Fuc-*O*-Ser/Thr, by B3GLCT in the ER (Sato et al. 2006). *O*-Fucose modification by POFUT2 and B3GLCT may play a role in the quality control of proteins containing TSRs, which require *O*-fucose glycosylation for their transport from the ER (Fig. 5.2) (Vasudevan et al. 2015). POFUT2 consists of two subunits, a

C-terminal domain and an N-terminal domain, with a large main cavity in the center of the subunit (Chen et al. 2012; Valero-González et al. 2016). Glycosylation is performed by pulling a TSR into this main cavity and a GDP-fucose into the small cavity on the C-terminal side of the main cavity. The glycosyltransferase reaction is activated by divalent metal ions such as magnesium, manganese, and calcium, but not by zinc (Chen et al. 2012). According to the structural analysis of *Caenorhabditis elegans* POFUT2, it has been shown that water molecules are important for glycosyltransferase activity, and that threonine residues are preferentially modified rather than serine residues (Valero-González et al. 2016). There are approximately 50 proteins with the consensus sequence for *O*-fucose transfer by POFUT2 (Schneider et al. 2017). It has been reported that ADAMTSL1, ADAMTSL2, ADAMTS9, ADAMTS13, TSP1, TSP2, F-Spondin, properdin, and punctin are modified with *O*-fucose transferred by POFUT2 (Vasudevan et al. 2015). Among them, POFUT2 is required for secretion in ADAMTS9 and ADAMTS13, and B3GLCT is also required for secretion in ADAMTSL1 and ADAMTSL2 (Vasudevan et al. 2015). The secretion of punctin is reduced by mutation of the *O*-fucosylation site by POFUT2 (Wang et al. 2007). *Pofut2* knockout mice showed a defect in primary gastrulation (Benz et al. 2016). This is due to the loss of function of ADAMTS9 in POFUT2 mutants (Du et al. 2010). This ADAMTS9 haplo-deficient mouse caused congenital corneal opacity and persistent lens-corneal adhesions called Peters' anomaly (Dubail et al. 2016). Proteins with TSRs are often modified by C-mannose as well as *O*-fucose, and notably, C-mannose is also proposed to play a role in the quality control of proteins containing TSRs (Shcherbakova et al. 2019).

Peters-Plus syndrome is characterized by abnormal eye development, forehead prominence, binocular isolation, short stature, and brachydactylia (Heinonen and Maki 2009; Holdener et al. 2020). Mutations in *B3GLCT* have been reported in patients with this disorder (Heinonen and Maki 2009). In mice, loss of *B3glct* resulted in symptoms of skeletal abnormalities, hydrocephalus, leukoplakia, soft tissue, and syndactyly, similar to Peters-Plus syndrome (Holdener et al. 2019). These symptoms are likely due to abnormalities in ADAMTS9 and ADAMTS20. However, sensitivity for the deficiency of *B3GLCT* is different between these two proteins. The symptoms of hydrocephalus and vitiligo in the *B3glct* mutant mice were caused by the loss of *Adamts20*. Abnormalities are caused by reduced *Adamts9*, and cleft palate is caused by *Adamts20* deficiency and reduced *Adamts9* function (Holdener et al. 2019).

5.3 *O*-Glucose Glycans

O-Glucose glycosylation is mediated by three protein *O*-glucosyltransferases, viz., POGLUT1-3 (Fig. 5.1). All these enzymes utilize UDP-glucose as a sugar donor substrate and EGF repeats as an acceptor substrate. POGLUT1 (Rumi in *Drosophila*) transfers *O*-glucose to a serine residue within a consensus sequence, $C^1XSX(P/A)C^2$, in EGF repeats (Acar et al. 2008; Takeuchi et al. 2011; Fernandez-Valdivia et al. 2011), whereas POGLUT2 and POGLUT3 transfer *O*-glucose to a serine residue within a consensus sequence, $C^3XNTXGSFXC^4$, in EGF repeats (Takeuchi et al. 2018a). X-ray structural analyses of *Drosophila* Rumi and human POGLUT1 have been performed, and the data show that POGLUT1 adds *O*-glucose to EGF repeats with very strict substrate specificity (Takeuchi et al. 2011, 2012; Li et al. 2017). POGLUT1 interacts with a wide range of amino acids in the acceptor EGF repeats in addition to the region between the first and second cysteine in the consensus sequence. Thus, POGLUT1 is able to transfer *O*-glucose to a properly folded EGF repeat only, which is consistent with the findings of biochemical assays (Takeuchi et al. 2012). Moreover, POGLUT1 is unique in that it modifies a serine residue, but not a threonine residue (Rana et al. 2011). The structure of POGLUT1 does not allow a glucose transfer to a threonine residue, which has a larger side-chain than a serine residue, because the POGLUT1 structure does not have enough space for interaction with the threonine

residue, unlike other *O*-linked glycosyltransferases (Li et al. 2017). POGLUT1 also utilizes UDP-xylose as a donor substrate, suggesting that it may function as a protein *O*-xylosyltransferase (Takeuchi et al. 2011). *O*-Glucose, transferred by POGLUT1, is elongated by two xylose residues in α-linkage, forming a trisaccharide, Xylα1-3Xylα1-3Glcβ1-*O*-Ser at most (Fig. 5.1). Recently, it has been reported that *O*-glucose attached to a subset of EGF repeats in NOTCH1 and NOTCH2 is elongated with a hexose and a sialic acid whose detailed structures have not been investigated to date (Urata et al. 2020). There are approximately 80 known proteins with the consensus sequence for *O*-glucose transfer by POGLUT1 (Rana et al. 2011). In mammals, GXYLT1 and GXYLT2 are responsible for xylose elongation at the non-reducing end of *O*-glucose (Sethi et al. 2010). In contrast to Fringe for *O*-fucose glycans described above, the biological reasons for two GXYLTs existing in mammals are not known. The enzyme responsible for the transfer of xylose to the non-reducing end of the Xyl-Glc disaccharide is XXYLT1. Interestingly, elongation of the *O*-glucose glycans is known to differ between different EGF repeats, even in the same protein (Sethi et al. 2012). This may mean that it is important to have a site-specific elongation level for each EGF repeat rather than for the entire protein (Urata et al. 2020). For example, mouse NOTCH1 receptor has 36 EGF repeats in the extracellular domain. However, in a reporter assay using NIH3T3 cells, DLL1-, and not JAG1-, dependent NOTCH1 signaling is reduced upon the introduction of a mutation at the *O*-glucosylation site at EGF28 (Rana et al. 2011). EGF28 in NOTCH1 is modified by *O*-glucose monosaccharides mainly in the same NIH3T3 cells (Takeuchi et al. 2017). These results suggest that *O*-glucose monosaccharide modification of EGF28 is required for DLL1-dependent activation of NOTCH1 (Fig. 5.3). It has not been reported that *O*-glucose transferred by POGLUT2 and POGLUT3 is elongated by other glycans. The *O*-glucosylation mediated by POGLUT2 and POGLUT3 is located in the region of human NOTCH1 that directly interacts with the ligand, and is in direct contact with the ligand DLL4 (Takeuchi et al. 2018a). Interestingly, this glycosylation at EGF11 of NOTCH1 appears to promote the transport of Notch to the cell surface, binding to ligands, and activation in concert with *O*-fucose glycosylation at EGF8 and EGF12 of NOTCH1 (Matsumoto et al. 2016; Takeuchi et al. 2018a). Further investigation of in vivo significance of POGLUT2- and POGLUT3-dependent *O*-glucosylation in Notch signaling, and possibly other EGF repeat-containing molecules, is currently underway (Takeuchi et al. 2018a).

The proteins known to be modified with *O*-glucose glycan are the Notch family, Notch ligands, Crumbs (Ramkumar et al. 2015), Eyes shut (Haltom et al. 2014), and peptidase domain-containing protein associated with muscle regeneration 1 (PAMR1) (Pennarubia et al. 2020a). The role of *O*-glucose glycans in the Notch family of proteins has been relatively well studied. In Notch, *O*-glucose monosaccharides are thought to be involved in protein quality control, improving EGF stability, facilitating transport to the cell surface (Fig. 5.2) (Takeuchi et al. 2017), and promoting signal transduction (Chu et al. 2013; Ishio et al. 2015). The effect of xylose elongation has also been shown. In *Drosophila*, the first xylosyl-extension of *O*-glucose glycans reduces the expression of Notch on the cell surface and suppresses Notch signaling (Lee et al. 2013). Conversely, although the extension of *O*-glucose glycans by the first xylose has no effect on the cell surface expression of endogenous NOTCH1 and NOTCH2 in human cells, it promotes Notch expression on the cell surface under Notch overexpression (Urata et al. 2020). These results suggest that the role of xylose in Notch signaling may differ among species. In the Crumbs family, the deletion of *O*-glucose glycans caused protein accumulation in the ER, especially in mouse Crumbs2 (Ramkumar et al. 2015). Thus, as with Notch, *O*-glucose glycans are important for the transport of Crumbs proteins to the cell surface. In *Drosophila*, Eyes shut is also modified with *O*-glucose glycans. The deficiency of *O*-glucose glycans in Eyes shut causes temperature-dependent intracellular accumulation of Eyes shut, leading to abnormal distribution of the

rhabdomere (Haltom et al. 2014). Thus, *O*-glucose glycosylation is important for protein transport in Eyes shut in *Drosophila*. In mice, embryos lacking *Poglut1* show an expanded neural version that does not fold properly (Fernandez-Valdivia et al. 2011). It has also been reported to cause severe growth retardation and abnormalities during development, including a lack of cardiac primordia and a shortened posterior axis, resulting in fetal death (Fernandez-Valdivia et al. 2011). It is also associated with cardiovascular phenotypes caused by abnormalities in Notch signaling (Fernandez-Valdivia et al. 2011). Severe cardiovascular damage, abnormal angiogenesis, lack of dorsal aorta and spina bifida, and bifurcation of the heart have been observed (Fernandez-Valdivia et al. 2011). Finally, the cardiac placenta did not unite, and the heart did not form. Defects were also present in vascular remodeling, with the formation of sinusoids, which differed from the arrangement of vessels normally observed (Fernandez-Valdivia et al. 2011). Defects were also present in endothelial remodeling (Fernandez-Valdivia et al. 2011). Molecularly, the protein level of JAGGED1 was increased in peri-portal vascular smooth muscle cells in *Poglut1* knockout mice (Thakurdas et al. 2016). *Poglut1* knockout mice had increased coronary arteries and bile ducts in the liver compared to the wild type. The increase in bile ducts is thought to be due to enhanced remodeling of bile duct epithelial cells by increased JAGGED1 (Thakurdas et al. 2016). It is not clear whether this change is Notch-dependent.

Muscular dystrophy is a general term for a disease with symptoms of skeletal muscle weakness and histological muscle changes. It is classified into five types, viz., laminin α2-related congenital muscular dystrophy, α-dystroglycan-related dystrophy, congenital disorders of glycosylation with abnormal α-dystroglycan glycosylation, collagen VI and integrin-related congenital muscular dystrophy, and intracellular and nuclear congenital muscular dystrophy (Falsaperla et al. 2016; Bönnemann et al. 2014). However, recently, patients with muscular dystrophy that do not fall into this category have been reported. Autosomal recessive mutations in *POGLUT1* were found in patients with muscular dystrophy (Servián-Morilla et al. 2016, 2020). The mutations were all missense mutations, some of which had reduced glycosyltransferase activity, and the others did not function correctly, such as those with a significant lack of protein stability. The α-dystroglycan from the patients' muscles, but not fibroblasts, lacked mature *O*-mannose glycans and reduced binding capacity to laminin, although it had a normal binding capacity to the ligand agrin (Servián-Morilla et al. 2016). Detailed structural analysis of *O*-mannose glycans from α-dystroglycan from patients will explain the differential effect on ligand specificity. The question of reduced POGLUT1 activity causing hypoglycosylation of α-dystroglycan still remains. Given the strict specificity of POGLUT1 toward properly folded EGF repeats with the *O*-glucose consensus sequence (Takeuchi et al. 2012), the absence of EGF repeats in α-dystroglycan suggests that the effect of the mutation of *POGLUT1* on glycosylation in α-dystroglycan is not direct, but indirect. In the patients' muscles, Notch signaling was reduced and satellite cells were also reduced. Satellite cells had decreased proliferative capacity and increased differentiation capacity. Thus, muscular dystrophy caused by mutations in *POGLUT1* may be due to the depletion of satellite cells. The depletion is due to excessive differentiation and decreased proliferation of satellite cells caused by reduced Notch signaling (Servián-Morilla et al. 2020). The reduced ability of α-dystroglycan to bind to ligands may be due to its reduced glycosylation. Since maturation of glycosylation of α-dystroglycan depends on the status of myogenesis (Goddeeris et al. 2013), abnormalities in the proliferation and differentiation of satellite cells due to reduced Notch signaling may cause hypoglycosylation of α-dystroglycan in the patients' muscles.

In addition to the aforementioned mutations in *POFUT1*, autosomal dominant mutations in *POGLUT1* have been found in patients with DDD. There are various types of mutations, including nonsense, missense, and frameshift mutations (Wilson et al. 2017; Basmanav et al. 2014; Kono et al. 2019; Strausburg et al. 2016;

Pavlovsky et al. 2018). The nonsense and frameshift mutations are expected to result in truncated forms of *POGLUT1* with little or no activity. Missense mutations are also thought to be important for donor-substrate interactions and to impair the enzyme's function at sites important for protein stabilization. Normal POGLUT1 was localized in the upper epidermis, but not in the patient's biopsy samples. It has been reported that loss of *POGLUT1* in cultured cells resulted in increased expression of *MITF*, which encodes a microphthalmia-associated gene and is known to be important for melanocyte development by regulating the expression of *TYR*, which encodes tyrosinase. These findings suggest that altered Notch signaling may be associated with increased levels of *MITF* and *TYR*, leading to increased melanin production and pigmentation (Ralser et al. 2019).

Elevated *POGLUT1* gene expression levels have been reported in many types of cancer, including primary cholangitis (Hitomi et al. 2019), breast (Jin et al. 2014), and colorectal cancer (Fang et al. 2017), T-cell acute lymphoblastic leukemia (Wang et al. 2010), acute myeloid leukemia (Wang et al. 2010), and non-small cell lung cancer (Chammaa et al. 2018). Reduced gene expression of *POGLUT1* has also been reported in patients with myelodysplastic syndromes (Karadonta et al. 2009). Mutations in *POGLUT1* have been reported in patients with hidradenitis suppurativa (Li et al. 2019), Moyamoya disease (Yang et al. 2020), and limb weakness (Töpf et al. 2020). High expression of POGLUT3 has been reported to correlate with poor prognosis in neuroblastoma (Tsai et al. 2020).

As for the xylosyltransferases that are responsible for the extension of *O*-glucose glycans, the gene expression of *GXYLT2* is downregulated in colorectal cancers adapted to a productive environment (colorectal cancer cells that can spread to the liver) (Zhou et al. 2019). In myeloid leukemia, it has been reported that ligand-dependent activation of Notch in leukemia cells enhances the expression of glycosyltransferases, *POFUT1*, *LFNG*, *MFNG, RFNG GXYLT1/2*, and *XXYLT1* (Wang et al. 2018). *GXYLT1* has been identified as a candidate causative gene for jugular paraganglioma (Snezhkina et al. 2019) and grossly abnormal pulmonary vein connection (Shi et al. 2018) and Parkinson's disease (Jia et al. 2020). Over-methylation of several genes, including *GXYLT2*, has been reported in cells with ulcerative colitis (Barnicle et al. 2017). *GXYLT2* has been identified as a genetic factor in caries development (Govil et al. 2018).

XXYLT1 has been reported to be amplified in many types of cancers, including lung squamous carcinoma (Yu et al. 2015). Of note, Notch signaling has been suggested to have a tumor-suppressive role in these cancers. Xylosyl-extension of *O*-glucose glycans in Notch suppresses Notch activation in a specific context in *Drosophila* (Lee et al. 2013). However, it is not clear whether xylosyl-extension of *O*-glucose glycans suppresses Notch activation in mammals and whether the amplification of *XXYLT1* is involved in tumor development. Genome-wide association studies have identified *XXYLT1* as one of the genes responsible for osteoporosis (Hu et al. 2018). Mutations in *XXYLT1* have been reported in patients with the oculo-auriculo-vertebral spectrum. This disease shows symptoms of auditory, oral, mandibular, and spinal developmental abnormalities, as well as heart, lung, kidney, skeletal, and central nervous system abnormalities (Guida et al. 2015). In patients with Chiari malformations (Guo et al. 2018), a defect in *XXYLT1* has been reported. It will be interesting to know whether the pathological mechanisms in these diseases are Notch-dependent or not.

5.4 *O*-GlcNAc Glycans

O-GlcNAc modification differs from *O*-linked glycosylation described above in that *O*-GlcNAc glycans can be divided into two types: intracellular and extracellular. Intracellular *O*-GlcNAc modification occurs on many proteins in the nucleus and cytoplasm (Levine and Walker 2016). Intracellular *O*-GlcNAc modification is transferred by a glycosyltransferase called OGT (Ju Kim 2020). Extracellular *O*-GlcNAc modifi-

cation was discovered more than 20 years later, after intracellular *O*-GlcNAc modification was discovered. Intracellular *O*-GlcNAc exists as a monosaccharide attached to proteins, while extracellular *O*-GlcNAc glycans attached to EGF repeats can be elongated. Here, we will focus on the extracellular *O*-GlcNAc modification. For more information about intracellular *O*-GlcNAc modification, please refer to the review articles published previously (Elbatrawy et al. 2020; Chatham et al. 2020; Ong et al. 2018; Nie and Yi 2019).

Extracellular *O*-GlcNAc modification is mediated by EGF-domain-specific *O*-GlcNAc transferase (EOGT), a glycosyltransferase that uses UDP-GlcNAc as a sugar donor substrate and EGF as a sugar accepting substrate (Fig. 5.1) (Sakaidani et al. 2012). Although three-dimensional structures of EOGT alone or in complex with any substrate have not been solved yet, EOGT appears to recognize the shape of EGF repeats as an acceptor substrate since it only utilizes correctly folded EGF repeats as an acceptor substrate like POFUT1 and POGLUT1 (Sakaidani et al. 2012). EOGT is an ER-localized enzyme, and *O*-GlcNAc transfer also takes place in the ER (Alam et al. 2020). The consensus sequence for *O*-GlcNAc addition is $C^5XXX(F/W/Y)(\underline{S/T})GXXC^6$ in EGF repeats (Ogawa et al. 2018). In the Golgi apparatus, galactosyltransferase(s) and sialyltransferase(s) elongate *O*-GlcNAc glycan by a galactose and a sialic acid, leading to the formation of Neu5Acα2-3/6Galβ1-4GlcNAcβ1-*O*-Ser/Thr trisaccharide (Sakaidani et al. 2012; Ogawa et al. 2018). The function of *O*-GlcNAc glycans has also been well studied in the context of Notch signaling, where *O*-GlcNAc glycosylation not only improves the protein stability of EGF repeats (Fig. 5.2), but also increases the ability of NOTCH1 to bind to the ligand DLL4 (Sawaguchi et al. 2017). It also enhances the DLL4-dependent signaling of NOTCH1 (Fig. 5.3) (Sawaguchi et al. 2017). Interestingly, although it enhances the interaction between DLL4 and NOTCH1, and its interaction-dependent signaling, it does not affect the interaction and signaling between NOTCH1 and JAGGED1 (Sawaguchi et al. 2017). Abnormalities in angiogenesis and retinogenesis, such as those observed in mice with haploinsufficiency of *Notch1* and *Rbpj,* and defective Notch signaling (Hellström et al. 2007; Kofler et al. 2015), are also observed in *Eogt* deficiencies in mice (Sawaguchi et al. 2017). Moreover, *Eogt* null mice showed impaired vascular maturation, increased vascular branching, and vulnerability to blood vessels compared to those in wild-type mice (Sawaguchi et al. 2017).

A mutation in EOGT has been reported in patients with Adams-Oliver syndrome (AOS) (Ogawa et al. 2015). EOGT with this mutation significantly reduced *O*-GlcNAc transfer activity and EOGT stability. This suggests that a reduced *O*-GlcNAc modification in some proteins causes AOS.

5.5 Perspectives

In this chapter, we outlined the *O*-linked glycans that modify the Notch receptors and regulate their functions. Ever since Fringe was reported to be a glycosyltransferase that modifies Notch (Moloney et al. 2000; Brückner et al. 2000), Notch signaling has become an important subject of study in glycoscience. Since then, new glycosyltransferases responsible for *O*-glycosylation and their biosynthesis have been identified. It has become clear that mutations and abnormalities in the expression of these genes cause a variety of diseases as summarized above. Similar discoveries are expected to continue in the future. The regulatory mechanism of Notch signaling by *O*-glycosylation needs to be fully understood. Furthermore, mass spectrometry is currently the most reliable method for detecting these low quantity and rare types of *O*-glycans, and many laboratories do not necessarily have specialized skills and access to expensive machines. Therefore, in the future, probes capable of specifically detecting these *O*-glycan modifications in in vivo tissues could be widely useful in the laboratory as well as in clinical practice.

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Compliance with Ethical Standards

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6 Extracellular Vesicles and Glycosylation

Yoichiro Harada, Yuki Ohkawa, Kento Maeda, Yasuhiko Kizuka, and Naoyuki Taniguchi

Abstract

Extracellular vesicles (EVs), a generic term for any vesicles or particles that are released from cells, play an important role in modulating numerous biological and pathological events, including development, differentiation, aging, thrombus formation, immune responses, neurodegenerative diseases, and tumor progression. During the biogenesis of EVs, they encapsulate biologically active macromolecules (i.e., nucleotides and proteins) and transmit signals for delivering them to neighboring or cells that are located some distance away. In contrast, there are receptor molecules on the surface of EVs that function to mediate EV-to-cell and EV-to-matrix interactions. A growing body of evidence indicates that the EV surface is heavily modified with glycans, the function of which is to regulate the biogenesis and extracellular behaviors of EVs. In this chapter, we introduce the current status of our knowledge concerning EV glycosylation and discuss how it influences EV biology, highlighting the potential roles of EV glycans in clinical applications.

Keywords

Extracellular vesicles · Exosomes · Glycosylation · Microvesicles · Tumors

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6.1 Introduction

Glycosylation describes a process in which glycan chains are generated on proteins and lipids, forming dense glycocalyx layers on the surface of cells, as well as on the luminal surface of endolysosomal compartments. Glycans have structural and biological roles and regulate a wide range of physiological and pathological events, including fertilization, development, differentiation, aging, neurodegenerative diseases, and tumor progression (Ohtsubo and Marth 2006). This functional diversity of glycans arises from their astronomical structural diversity defined by the aglycon moiety, monosaccharide composition, anomeric linkage, and branching. These heterogeneities are generated sophisticatedly by the concerted action of glycosyltransferases, glycosi-

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dases, chaperones, and transporters of nucleotide sugars, ions, and metals, which can be further controlled by their gene expression, localization, and the supply and competition of substrates (Taniguchi and Kizuka 2015; Kizuka and Taniguchi 2016; Pinho and Reis 2015).

More than half of all newly synthesized proteins in the secretory pathway are modified with glycans (Apweiler et al. 1999). While glycoproteins pass through the secretory pathway and are delivered to their final destination, the glycan moieties undergo dramatic structural remodeling to control the quality, stability, and functionality of the carrier proteins. As glycoproteins age, they are delivered to lysosomes for degradation. The tight regulation of the birth and death of glycoproteins is essential for the maintenance of systemic homeostasis, as evidenced by numerous studies concerning congenital disorders of glycosylation and lysosomal storage diseases (Freeze et al. 2015a, 2015b). Alterations in cell surface glycosylation are also frequently observed in many diseases, for example, chronic inflammation, malignant transformations, type II diabetes, and neurodegenerative diseases (Pinho and Reis 2015; Ohtsubo et al. 2011, 2005; Gao et al. 2017; Kanekiyo et al. 2013; Kizuka et al. 2016). On these bases, glycobiology research has extensively focused on elucidating the structural complexity and biological relevance of glycans on and inside cells.

Meanwhile, cells make a large effort to release membranous vesicles that are heavily coated with glycans (Fig. 6.1a) (Williams et al. 2018; Moreno-Gonzalo et al. 2014). These vesicles, termed extracellular vesicles (EVs), constitute a large family of secretomes and play an important role in the disposal of cellular waste and the dissemination of biologically active macromolecules (Mathieu et al. 2019; LeBleu and Kalluri 2020; Kalluri and LeBleu 2020; Stahl and Raposo 2019). These molecules include nucleotides, cytosolic/nuclear proteins, membrane proteins, and lipids, none of which can be secreted through the conventional secretory pathway. EVs are currently classified into three major classes depending upon their origin, size, and cargo proteins: Plasma membrane-derived microvesicles that are enriched in annexin A1 (100–1000 nm), endosome-derived exosomes that are enriched in tetraspanin proteins (50–150 nm), and distinct nanoparticles/exomeres that are enriched in proteins that are involved in glycolysis and the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling (< 50 nm) (Fig. 6.1b) (Kowal et al. 2016; Jeppesen et al. 2019; Zhang et al. 2018, 2019). The cargo profiles of each one of these EV subfamilies can differ depending upon the cell types that produce them and the cellular states, thus generating considerable vesicle heterogeneity in the extracellular space. Such EV heterogeneity is thought to contribute to EV functions in modulating a large number of biological and pathological processes, including development, differentiation, aging, thrombus formation, immune responses, neurodegenerative diseases, and tumor progression.

An emerging concept that EVs shuttle their cargo molecules, especially messenger RNAs, micro RNAs, and membrane proteins, between cells to transmit signals has significantly improved our understanding of EV biology, while the importance of glycosylation in the biogenesis, extracellular behavior, and functions of EVs remains largely elusive. In this chapter, we summarize our current view of the classification,

Fig. 6.1 (continued) GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; IdoA, iduronic acid; GlcN, glucosamine. (**b**) Classification of EVs. Cells secrete at least three vesicle types: Microvesicles (100–1000 nm), exosomes (50–150 nm), and exomeres (<50 nm). Microvesicles are shed directly from the plasma membrane and are characterized by the presence of annexin A1. In contrast, exosomes, which carry tetraspanin proteins (CD9, CD63, and CD81), are secreted via membrane fusion between the plasma membrane and multi-vesicular endosomes, with the intraluminal vesicles being released as exosomes. Exomeres, which are enriched with proteins involved in glycolysis and the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling, have recently been identified as distinct nanoparticles of unknown cellular origin. Microvesicles and exosomes can be enriched by sequential centrifugation at 10,000–20,000 × *g*, followed by 100,000 × *g*. However, this procedure cannot fully separate microvesicles and exosomes, so that the 10,000–20,000 × *g* pellets and the 100,000 × *g* pellets are referred to as large EVs and small EVs (sEVs), respectively

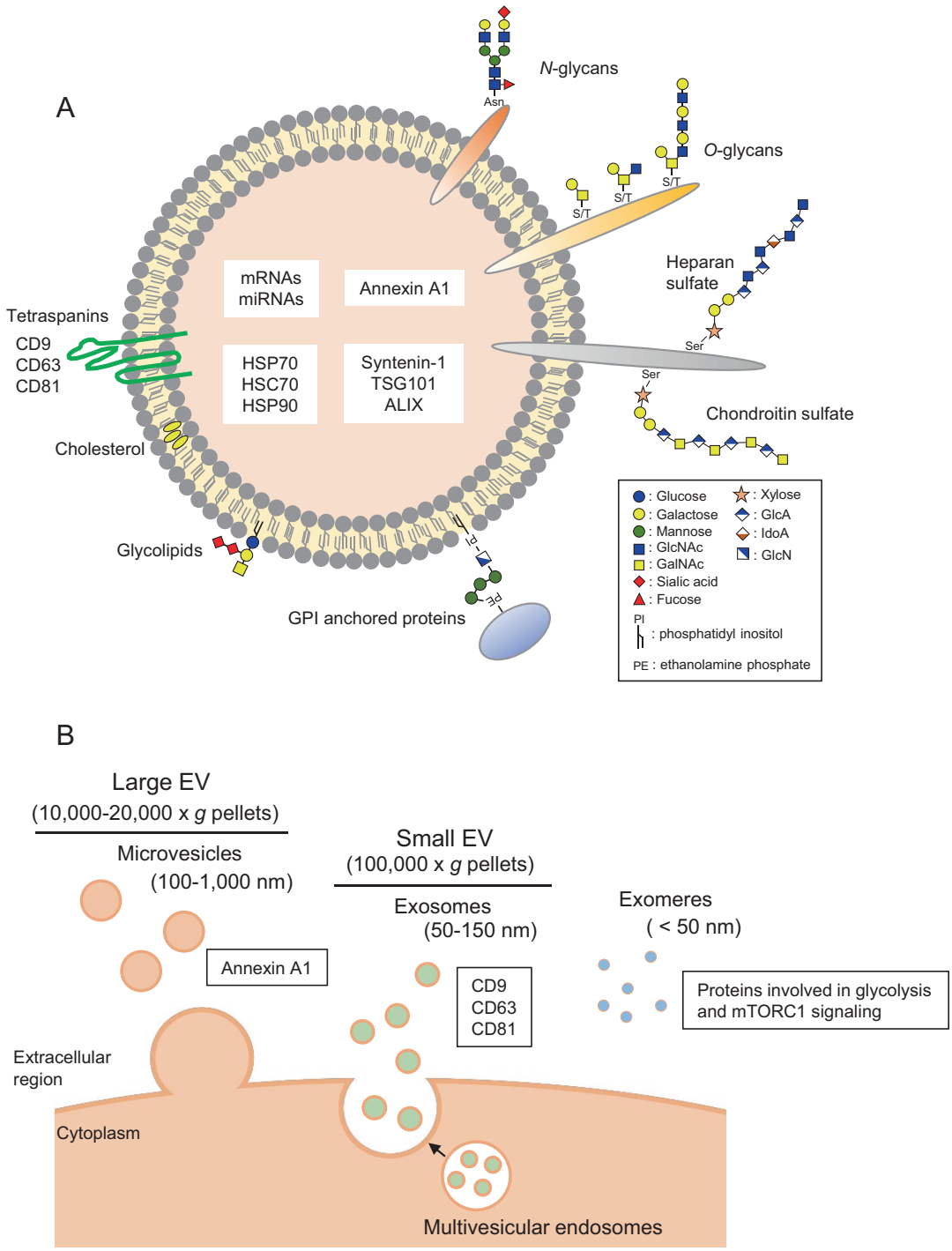

Fig. 6.1 The composition and classification of EVs. (**a**) Compositions of EVs. The lumen of EVs contains nucleotides [messenger RNAs (mRNAs) and micro RNAs (miRNAs)] and cytosolic/nuclear proteins. The EV membranes contain various lipids (phospholipids, lysophospholipids, cholesterol, ceramide, sphingomyelin, and glycosphingolipids), glycosylphosphatidylinositol (GPI)-anchored proteins, and membrane proteins modified with *N*-glycans, *O*-glycans, and glycosaminoglycans (heparan sulfate and chondroitin sulfate). GlcNAc, *N*-acetylglucosamine;

biogenesis, and functions of EVs, and then discuss how glycosylation comes into the picture of EV biology.

6.2 Classification, Biogenesis, and Functions of EVs

Exosomes are one of the very first discovered and best studied EVs. In 1983, Rose Johnstone and Clifford Harding independently identified exosomes as a carrier of the transferrin receptor that is released during reticulocyte maturation (Pan and Johnstone 1983; Harding et al. 1983). In maturing reticulocytes, the transferrin receptor becomes engaged with multivesicular endosomes, where the receptor is incorporated into intraluminal vesicles (ILVs) by the inward budding of endosomal membranes. The multivesicular endosomes rarely fused with lysosomes for degradation but they readily fused with the plasma membrane to release ILVs in the form of exosomes, providing a rationale for the rapid clearance of the transferrin receptor from maturing reticulocytes. Exosome secretion is not limited to reticulocytes but rather is a general phenomenon found in most, if not all, vertebrate and invertebrate cells. Among the EVs, it has been proposed that exosomes are vesicles that carry tetraspanin proteins (i.e., CD9, CD63, and CD81) (Kowal et al. 2016; Jeppesen et al. 2019). Exosomes are further flavored with unique molecular patterns, originating upon the cells that produced them, which confer diverse functions to exosomes. As a waste vessel, exosomes are involved in the secretion of cytotoxic materials, such as intrinsic or viral DNA fragments and the amyloid β and tau proteins (Takahashi et al. 2017; Oikawa et al. 2009; Asai et al. 2015). However, exosome-associated tau can be transmitted to microglia to facilitate undesirable tau propagation. As system for the delivery of signaling molecules, exosomes are involved in the modulation of immune responses and tumor progression (Becker et al. 2016; Robbins and Morelli 2014). For example, antigen-presenting cell-derived exosomes directly or indirectly induce antigen-specific T cell responses (Raposo et al. 1996; Thery et al. 2002), while exosomes that are released from activated cytotoxic $CD8^+$ T cells prevent the progression of fibroblastic tumors by ablating mesenchymal tumor stromal cells (Seo et al. 2018). However, tumor cells attempt to survive and proliferate for metastasis by secreting a large amount of exosomes that carry pro-angiogenic, pro-metastatic, and immune modulatory molecules (Becker et al. 2016; Chen et al. 2018; Poggio et al. 2019).

Microvesicles, which shed directly from the plasma membrane, also constitute a large class of EVs. The biogenesis of microvesicles requires complex intracellular signaling networks that control cytoskeletal remodeling to induce membrane protrusion and neck contraction of the forming vesicles. Microvesicles are also known to mediate the disposal and horizontal transfer of the cargo molecules. However, microvesicles and exosomes have different molecular compositions and are currently thought to be involved in distinct, but partially overlapping biological processes because their molecular compositions and the sites of biogenesis are different. The release of exosomes occurs relatively constantly, while that of microvesicles can be induced particularly during apoptosis and platelet activation, further highlighting the functional differences between these two EV types.

EVs are widespread in body fluids (e.g., blood, urine, breast milk, and saliva), as well as in culture supernatants of mammalian cells. Classically, EVs are separated from these specimens into microvesicles as 10,000–20,000×*g* pellets (100–1000 nm in size) and exosomes as 100,000×*g* pellets (50–150 nm in size) by sequential centrifugation (Fig. 6.1b). The low- and high-speed pellets are often referred to as large EVs and small EVs (sEVs), respectively, because the centrifugation procedure does not completely separate microvesicles and exosomes (Thery et al. 2018). It should also be noted that we still do not see an overall picture of the EV heterogeneity, since novel EV types have been increasingly discovered or defined (e.g., exomeres, small exosome vesicles, and large exosome vesicles) (Zhang et al. 2018, 2019). In this chapter, we refer microvesicles and exosomes as large EVs

and sEVs, when they were prepared by ultracentrifugation. Otherwise, we specify the vesicle or particle types.

6.3 Roles of Glycosylation in EV Biogenesis and Cargo Sorting

The biogenesis of EVs is a complex, but tightly regulated process that is initiated by the sorting of cargo molecules into limiting membranes, followed by the deformation of the membrane domains and closure of the necks of the forming vesicles (Fig. 6.2). Five major molecular mechanisms have been proposed for the formation of EVs, namely, endosomal sorting complexes required for transport (ESCRTs) (Colombo et al. 2013), neutral sphingomyelinase 2 (nSMase 2) (Trajkovic et al. 2008), tetraspanins (Andreu and Yanez-Mo 2014; van Niel et al. 2011), ADP-ribosylation factor 6 (ARF6) (Muralidharan-Chari et al. 2009; Ghossoub et al. 2014), and the arrestin domain containing protein 1 (ARRDC1) (Nabhan et al. 2012). Some of these pathways are known to function together in EV biogenesis. For example, the ALG-2 interacting protein X (ALIX), an accessory protein of ESCRT-III, associates with the syndecan–syntenin complex and drives exosome biogenesis with ARF6 (Baietti et al. 2012; Ghossoub et al. 2014). This process is further controlled by the action of heparanase, which internally digests heparan sulfate chains on syndecan proteins and elicits their aggregation and proteolytic cleavage, triggering the formation of ILVs in multivesicular endosomes by ESCRTs (Roucourt et al. 2015) and the release of exosomes.

Microvesicle shedding from the plasma membrane involves the ARF6-phospholipase D

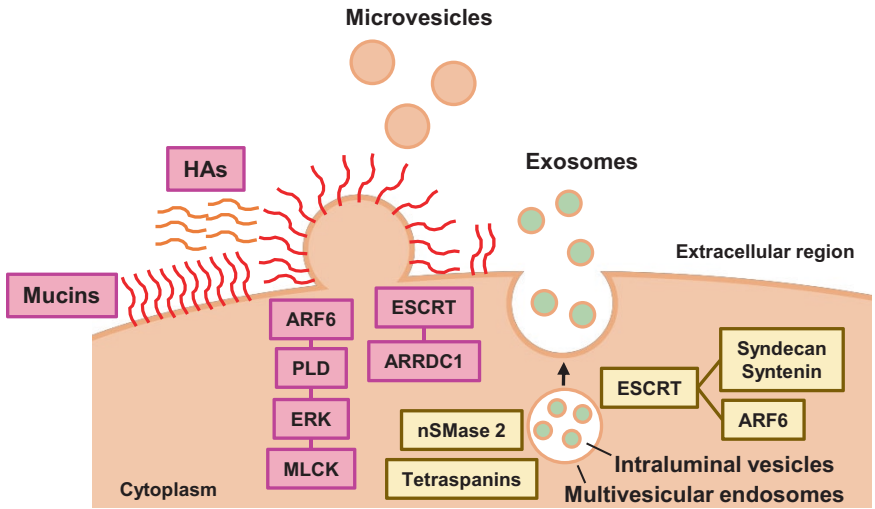

Fig. 6.2 The current model of EV biogenesis. Exosomes are originally formed as intraluminal vesicles by the inward budding of the endosomal membrane. This process is driven by several mechanisms, including the endosomal sorting complexes required for the transport (ESCRT) machinery, neutral sphingomyelinase 2 (nSMase 2), and tetraspanins. The ESCRT machinery is known to function cooperatively with the syndecan-syntenin complex to facilitate the biogenesis of the ADP-ribosylation factor 6 (ARF6)-dependent exosome. Microvesicles are formed by the outward budding of the plasma membrane, which is driven by the ESCRT machinery or ARF6-dependent signaling pathway. The arrestin domain containing protein 1 (ARRDC1), which binds to the tumor susceptibility gene 101 (TSG101), an essential subunit of ESCRT, is critical for the formation of ARRDC1-mediated microvesicles (ARMMs; ~100 nm). ARF6 is a small GTPase that activates phospholipase D (PLD). This facilitates the extracellular signal-regulated kinase (ERK)-dependent phosphorylation of the myosin light chain kinase (MLCK), triggering acto-myosin contraction at the neck of the forming vesicles. Mucins and hyaluronic acids (HAs) form a glycocalyx on the surface of cells which facilitates membrane deformation and the budding of microvesicles

(PLD)—the extracellular signal-regulated kinase (ERK) signaling axis that activates the myosin light chain kinase (MLCK) to facilitate the release of microvesicles by acto-myosin-mediated neck contraction of the forming vesicles (Muralidharan-Chari et al. 2009). It has been shown that the dense accumulation of mucins or hyaluronic acids on the plasma membrane generates sufficient forces to alter the shape of the membrane, which then induces membrane projection and blebbing and facilitates microvesicle release (Shurer et al. 2019). This process is further promoted by disassembly of the F-actin lining membrane projection, indicating that cytoskeletal organization and the extracellular glycocalyx cooperate to regulate microvesicle shedding at the plasma membrane.

Many membrane proteins that are expressed on the EV surface are modified with *N*-glycans, thus playing a crucial role in the sorting of the carrier proteins into sEVs. EWI-2 (immunoglobulin superfamily protein 8) is a glycoprotein that contains three potential *N*-glycosylation sites in humans and is secreted via sEVs (Liang et al. 2014). It has been shown that all three *N*-glycosylation sites of EWI-2 are required for maximal secretion but they are not required for proper cellular localization of this protein. Further, the processing of oligomannose-type to complex-type *N*-glycans of some glycoproteins (e.g., EWI-2, CD63 and LGALS3BP) is involved, at least in part, in the sorting of these glycoproteins into sEVs (Liang et al. 2014; Gomes et al. 2015). Since more than half of all proteins synthesized in the secretory pathway are modified with *N*-glycans, *N*-glycosylation may mediate the sorting of a large number of glycoproteins into sEVs.

6.4 Glycosylation of EVs

A pioneering work on elucidating the EV glycome by using a lectin microarray was first reported from Mahal's group. They found that the glycomes of human immunodeficiency virus-1 (HIV-1) strains are very similar to that for sEVs, which they referred to as microvesicles in their studies, and that they are released from the matched uninfected T cell lymphoma H9 cell line, possibly allowing the virus to escape from the host immune system (Krishnamoorthy et al. 2009). The glycan signatures of HIV-1 and sEVs from the same producing T cells were characterized by an enrichment in oligomannose-type and multi-antennary complex-type *N*-glycans and sialylation, as well as the exclusion of blood group A and B antigens. Interestingly, lectin profiling of the producing T cells was not completely matched to that of HIV-1 or sEVs, suggesting that these particles are formed from sites where specific glycoforms and/or glycoconjugates are highly enriched. The similarity of lectin-profiling data between sEVs that are released from various tumor cell lines was also proven (Batista et al. 2011; Shimoda et al. 2017), suggesting that oligomannose-type and multi-antennary complex-type *N*-glycans and hyper sialylation are probably a conserved glycan signature of tumor cell-derived sEVs (Batista et al. 2011), despite the fact that the carrier glycoconjugates can be cell type-specific. In contrast, discrete glycan signatures can also be formed on the surface of sEVs, depending upon the states of the cells that are producing them. For example, an agglutinin from the freshwater cyanobacterium *Oscillatoria Agardhii* (OAA), which strongly binds to oligomannose-type *N*-glycans, can capture CD81-positive sEVs derived from skin, lung, and brain cancer cell lines, but not those from non-tumorigenic cells (i.e., human endothelial cells or dermal fibroblasts) (Yamamoto et al. 2019). Moreover, the recombinant form of the lectin derived from *Burkholderia cenocepacia* (rBC2LCN) preferentially marks sEVs that are released from undifferentiated human-induced pluripotent stem cells (hiPSCs) over those released from non-hiPSCs [i.e., human chondrocytes, dermal fibroblasts, and adipose tissue-derived mesenchymal stem cells (MSCs)] (Saito et al. 2018). Further, the differentiation of human adipose tissue-derived MSCs into osteogenic cells generates a unique glycome on their sEVs (Shimoda et al. 2019). All of these findings underscore lectins as an attractive tool for developing EV glycome-based biomarkers in the qual-

ity control of stem cell therapy and the detection of tumors.

In practice, the heterogeneity of EVs matters since there are many procedures for EV preparation (i.e., ultracentrifugation, polymer-based precipitation, affinity capturing and so on), the protocols of which could further vary depending upon the researcher. Furthermore, no definitive marker molecules and detection/quantification methods for individual vesicle types are currently available (Jeppesen et al. 2019; Kowal et al. 2016), thus hampering our understanding of the biological functions of EVs. However, it has been proposed that bona fide exosomes contain tetraspanin proteins (i.e., CD9, CD63, and CD81), but not all exosomes appear to contain all three tetraspanins (Kowal et al. 2016). A major breakthrough in dissecting such exosome heterogeneity occurred by the immuno-isolation of exosomes with anti-CD9, anti-CD63, and anti-CD81 antibodies, followed by their in-depth proteomics analysis (Kowal et al. 2016). A recent study by Matsuda et al. employed this immuno-isolation procedure and lectin microarray to compare glycan profiles between exosome subpopulations, revealing that CD81-positive exosomes have different glycomes from CD9-positive or CD63-positive exosomes in several pancreatic cancer cell lines (Matsuda et al. 2020). The CD81-positive exosomes react poorly with the isolectin L4 from *Phaseolus vulgaris* (PHA-L4) but react highly with the lectin from *Lycopersicon esculentum* (LEL). These two lectins are known to recognize *N*-glycan structures that are associated with tumor growth, invasion, interaction with extracellular matrix, angiogenesis, and metastasis (Cummings and Kornfeld 1982; Yoshimura et al. 1995; Granovsky et al. 2000; Croci et al. 2014), and these tumor-associated processes are known to be facilitated by membrane glycoproteins (e.g., integrins and receptor tyrosine kinases) that are displayed on tumor cell-derived EVs (Becker et al. 2016). It is therefore tempting to speculate that the glycosylation of these proteins may have a critical role in modulating EV biogenesis, as well as the pro-malignant and pro-metastatic potentials of tumor cell-derived EVs.

6.5 Roles of EV Glycans in Cellular Recognition and Uptake

Although little is known regarding the biological function of EV glycans, emerging evidence suggests that they affect the cellular uptake of EVs. Adipose tissue-derived mesenchymal stem cells (ADSCs) secrete sEVs that are highly reactive with lectins that are specific for Siaα2,6Gal (Shimoda et al. 2017). When mice are subcutaneously injected with ADSC-derived sEVs, the sialic acid moiety of EV glycans functions as a ligand for sialic acid-binding immunoglobulin (Ig)-like lectins (Siglecs) that are expressed on CD11b-positive antigen presenting cells in lymph nodes. The interaction between Siglecs and EV glycans also mediates, at least in part, the uptake of ADSC-derived sEVs by these cells. Similar to this observation, sEVs that are released from glioblastoma cells contain glycans that are capped with sialic acids, which strongly bind to Siglec-9 in vitro but the sEVs are rarely taken up by human monocyte-derived dendritic cells (moDCs) (Dusoswa et al. 2019). Interestingly, the introduction of an artificial glycolipid carrying a Lewis^{Y} moiety into glioblastoma-derived sEVs strengthens the binding of the sEVs to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), improving the uptake of the sEVs by moDCs (Dusoswa et al. 2019). In addition, the simultaneous enzymatic digestion of sialic acids and *O*-glycans, but not *N*-glycans, on human breast cancer cell-derived sEVs increases the lung tropism of the sEVs (Nishida-Aoki et al. 2020). Similarly, the enzymatic removal of sialic acids from mouse hepatic cell-derived sEVs has been shown to improve the in vitro uptake of the modified sEVs by various cell types, including skin, lung, retinal, ovarian, and prostate cells (Williams et al. 2019). This increase in the cellular uptake of sEVs upon sialic acid removal is most likely due to the reduction in steric hindrance and/or electrostatic repulsion between the negatively charged surfaces of sEVs and the recipient cells. These collective findings raise the possibilities that EVs with different glyco(proteo)mes or cell

origins may have distinct tropisms at the systemic or tissue levels (Hoshino et al. 2015; Zhang et al. 2018; Rodrigues et al. 2019), and that designer EVs carrying various glycosylation patterns may be useful in terms of controlling organ/tissue specificities and cellular uptake in pharmaceutical applications.

Glycan-modifying enzymes (i.e., glycosidases and glycosyltransferases) are also present on the surface of EVs of various cellular origins. Lipopolysaccharide (LPS)-stimulated microglial cells secrete the lysosomal sialidase Neu1 via sEVs, thus facilitating the degradation of cell surface polysialic acids for the release of preexisting neurotrophin (Sumida et al. 2015). The addition of α2,6-linked sialic acids to the terminal galactose residues of *N*-glycans is catalyzed mainly by the β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) enzyme. This sialyltransferase is present as an active form in sEVs and exomeres, which are very small, recently identified non-membranous particles (<50 nm) (Zhang et al. 2018, 2019). Strikingly, the transfer of sEV- or exomere-associated ST6GAL1 from tumor cells to the recipient cells results in hypersialylation of the cell surface β1 integrin (Zhang et al. 2019), possibly contributing to tumor cell motility and invasion (Seales et al. 2005; Zhu et al. 2001; Christie et al. 2008; Woodard-Grice et al. 2008). These collective findings strongly suggest that glycans and glycan-modifying enzymes on EVs modulate more widespread biological processes than originally anticipated.

6.6 EV Glycoproteome as a Potential Target for the Development of Tumor Biomarkers

Tremendous efforts have been made to identify EV proteins associated with tumor development, progression, and metastasis. One possible straightforward strategy for achieving this is prospective studies, that is, comparing EV proteomes between controls and tumor patients. However, a major drawback to this strategy is the isolation of total, but not disease-specific, EVs from complex biological fluids (e.g., plasma, serum, and urine), which also contain abundant amounts of soluble (glyco)proteins that are not associated with EVs and can be contaminants in the EV preparation. To overcome these issues, several studies have taken advantage of using the non-tumorigenic and tumor cell lines from different origins or with distinct metastatic potentials as EV sources. Assembling and comparing proteomes of EVs that are released from these cell lines have highlighted tumor-associated/specific EV proteins, including hepatocyte growth factor receptor (MET), tyrosinase-related proteins 2/L-dopachrome tautomerase (Peinado et al. 2012) and the programmed cell death 1 ligand 1 (PD-L1) (Chen et al. 2018) in metastatic melanomas, glypican-1 in patients with pancreatic ductal carcinomas or pancreatic cancer precursor lesions (Melo et al. 2015), integrin αV in liver metastasis of pancreatic cancers, and integrin β4 in lung metastasis of various tumors (Hoshino et al. 2015). Strikingly, all of these identified proteins are *N*-glycoproteins and, importantly, the *N*-glycans of these proteins have functional roles when they are expressed in cells (Kariya and Gu 2011; Pochec et al. 2015; Svensson et al. 2011; Awad et al. 2015; Hsu et al. 2018; Li et al. 2018). It would be interesting to determine the glycan structures on tumor-specific, EV-associated glycoproteins in an attempt to investigate their diagnostic and prognostic values, as well as their functional roles in the development and progression of various tumors.

6.7 Concluding Remarks

Glycocalyx is a structurally and functionally important matrix covering the surface of cells, as well as EVs. These vesicles share a common glyco(proteo)me with the cells that produce them, while it is becoming crystal clear that EVs are enriched with selected glycoconjugates, glycan structures, and glyco-related enzymes, creating non-cell autonomous, unique bioactive membrane fields on EVs. There are several interesting questions raised. First, what factor(s) determines the glyco(proteo)me during EV bio-

genesis? To be packaged into vesicles, the cargo molecules need to be trafficked to the vesicle forming sites, which possibly involve master regulators that mediate the bulk sorting of cargo glycoconjugates or specific sorting signals given to each one of cargo glycoconjugates. The glycocalyx formed on the EV surface in this manner represents the first contact sites for interacting with the extracellular environment and surrounding cells. What then could be the physiological and pathological functions of EV glycans? As a whole, bulky glycans form a repulsive, hydrophilic shield on EVs and may protect functional epitopes from the action of proteases or glycosidases until the EVs operate their jobs. Glycans can also fine-tune the trafficking, localization, and turnover of carrier molecules in cells. What then would be the fate of EV-associated glycoconjugates and glyco-related enzymes after they are delivered to the recipient cells? Are these cargos simply degraded in lysosomes upon delivery or do they remain functional at the sites where they are supposed to function? Addressing these fundamental questions will open up new avenues of EV biology and clinical applications.

Disclosure of Interests The authors declare no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Quantitative Genetics of Human Protein *N*-Glycosylation

7

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Abstract

Although changes in protein glycosylation are observed in a wide range of diseases and pathological states, the examples of use of glycans as biomarkers and therapeutic targets are limited. This is not in small part because the understanding of human glycome regulation in vivo is incomplete and fragmented. Combination of human glycomics and genomics offers a powerful "data-driven hypotheses" approach to dissect the complex human glycobiology in vivo in an agnostic manner.

In this chapter we review a decade of quantitative genetic studies of human *N*-glycome, including studies of its heritability and gene-mapping via genome-wide association studies (GWASs). We show that GWASs of human *N*-glycome start revealing regulators of the biochemical network of *N*-glycosylation. Some of these regulators demonstrate pleiotropic effects on human disease, especially autoimmune and inflammatory. We emphasize the use of in silico functional methods and multi-omics approaches to prioritize functional mechanisms to be further validated in laboratory experiments. This combined approach will lead to better understanding of mechanisms of regulation of human protein glycosylation and will provide a rich source of etiologic insight, therapeutic interventions, and biomarkers.

Keywords

Genetic regulation · Heritability · Genome-wide association · *N*-glycosylation · Glycoproteins · Glycomics

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7.1 Introduction

The large observed intra- and inter-individual differences in protein *N*-glycosylation are increasingly recognized to be implicated in human pathologies. Beyond that, aberrant glycosylation is involved in key pathological steps of tumor development and is even considered a new hallmark of cancer (Vajaria and Patel 2017; Munkley and Elliott 2016; Taniguchi and Kizuka 2015). Glycans are considered as potential therapeutic

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targets (RodrÍguez et al. 2018) and biomarkers for early diagnosis and disease prognosis (Thanabalasingham et al. 2013; Adamczyk et al. 2012; Shinohara et al. 2015; Peng et al. 2018), which makes glycobiology a promising field for future clinical applications.

Protein glycosylation is a complex process depending on the interplay of multiple enzymes catalyzing glycan transfer, glycosidic linkage hydrolysis as well as glycan biosynthesis. Abundance of specific protein glycoforms can be influenced by a variety of parameters including activity of enzymes and availability of substrates, accessibility of a glycosylation site, protein synthesis, and degradation. Overall, protein glycosylation is controlled by genetic, epigenetic, and environmental factors (Lauc et al. 2014; Moremen et al. 2012; Knezevic et al. 2009). Although biochemically, glycosylation pathway is well-studied (Hashimoto et al. 2006), little is known about networks of genes that orchestrate cell- and tissues-specific regulation of these biochemical reactions in humans in vivo.

How can this daunting task be approached? One of the ways to acquire new biological knowledge is through "data-driven hypotheses" research, in which omics technologies and the large data sets they produce help scientists formulate better and more relevant hypotheses to be put to the test (Van Helden 2013). Such research relies on collection of (a large) sets of samples and their thorough molecular characterization in an unbiased manner. This requires availability of high-throughput technologies that can assess the domain of interest. One of the possible ways for data-driven hypotheses research in human glycobiology is through a combination of glycomics and genomics. Here, "glycomics" refers to an unbiased study of a spectrum of glycans present in living systems and "genomics" refers to a set of techniques that allow dissection of complex biological phenomena.

Generally speaking, the glycome comprises all the glycan structures synthesized by an organism (Henrissat et al. 2017). It is analogous to the genome, the transcriptome, and the proteome, but is thought to be even more dynamic and having higher structural complexity. Cells of different types will synthesize a subset of the glycome based on their differentiation state and physiological environment. Here, we will use the term "glycome" to denote a quantified repertoire of glycans that a cell or tissue produces under specified conditions of time, location, and environment. We will also use the term "single-protein glycome" in application to a quantified spectrum of glycans attached to a specific protein.

Much of what we know from large cohort studies of human *N*-glycosylation was learned from studies of glycome of blood plasma (plasma *N*-glycome), that is the spectrum of glycans attached to the blood plasma glycoproteins; and from the *N*-glycome of the immunoglobulin G (IgG glycome). In particular, association with changes in plasma *N*-glycome or IgG glycome has been found for Parkinson's disease (Russell et al. 2017), low back pain (Trbojević-Akmačić et al. 2018), rheumatoid arthritis (Gudelj et al. 2018b), ulcerative colitis, Crohn's disease (Trbojević Akmačić et al. 2015), type 2 diabetes (Lemmers et al. 2017), and others (see Gudelj et al. 2018a for a review). It was demonstrated that IgG glycosylation strongly changes with age (Krištić et al. 2014). Although changes in different glycan abundances were robustly associated with various diseases, only a limited number of glycan structures are established as diagnostic biomarkers or therapeutic targets. This is in large part because interpretation of observed disease-associated glycan changes seen in various diseases is limited by our incomplete knowledge about glycosylation processes and their regulation.

Classical experimental science approach assumes investigation of complex systems by introducing perturbations in these systems and by tracking the consequences of these perturbations. Study of human biology is difficult because possibilities for such classical experimentation are rather limited. A wide range of model systems are used as proxies in a hope to capture certain aspects of human biology. However, such systems often fail to reflect these peculiarities of human biology that are very important. This especially concerns a study of multifactorial traits that arise from a complex interplay of a

multitude of genetic and environmental factors. On the other hand, epidemiologic observational and intervention studies, although having an advantage of investigating humans directly and not via a proxy, are expensive, time-consuming, and rather restricted in terms of introducing perturbations in a controlled way.

In this context, genetics offers an attractive approach to study of human biology in vivo by piggybacking on the experiments of nature. More specifically, the inherited genetic variation, the specific genetic make-up that distinguishes one individual from another already at the conception, could be considered as a set of perturbations introduced (inherited) at the very start of the development. Tracking the consequences of these perturbations helps understanding human biology by, on one hand, linking DNA changes to a trait of interest, and, on the other hand, by understanding what are the functional consequences of this DNA change. Nowadays, the first step of this "tracking"—namely, linking a genetic variant to a phenotype—is done using two dominant study designs. The first is a study of affected families where a rare Mendelian mutation exhibiting a large effect segregates. The second is a study of subtle effects of common genetic variation onto the traits of interest in the framework of a genome-wide association study (GWAS) that typically includes thousands of participants. In application to glycobiology, the first approach is used to study congenital disorders of glycosylation. This topic is beyond the scope of this chapter. The second approach is used to study the effects of common variants, e.g., single nucleotide polymorphisms (SNPs), on relative abundances of glycan structures that are measured using high-throughput techniques that allow profiling of thousands of samples.

In this chapter, we will first review what we know from the biochemical and model studies about human genes encoding enzymes involved in *N*-glycosylation. We then will review results of human quantitative genetic studies of protein *N*-glycosylation in large cohorts and will summarize what we have learned from these studies. Finally, we will discuss future perspectives in human quantitative glycogenomics.

7.2 Genes Involved in the Protein *N*-Glycosylation Pathway

A collection of over 900 human genes (Cummings and Pierce 2014) and approximately the same number of mouse genes (Nairn et al. 2008; Cummings and Pierce 2014) has been designated as being related to glycosylation. Given the total number of genes in humans—that is approximately 20,000—it follows that around 5% of all human genes are associated with glycosylation. These glycosylation-related genes (which we will call "glycogenes") include not only genes that encode enzymes and other proteins involved in synthesis and catabolism of glycans but also genes that encode proteins involved in recognition and binding of specific glycans (i.e., glycan-binding proteins) and genes that encode different core proteins of proteoglycans (Nairn et al. 2008; Cummings and Pierce 2014). Also, to avoid confusion, it should be clarified here that this number of more than 900 human glycogenes comprises not only genes involved in the protein *N*-glycosylation pathway but also genes involved in other human glycosylation pathways, including the protein *O*-glycosylation pathway, glycolipid synthesis pathway, glycosaminoglycan biosynthesis pathway, and GPI-anchor biosynthesis pathway (Nairn et al. 2008; Cummings and Pierce 2014).

The biosynthesis of *N*-glycans attached to proteins is not directed by a genetic template as is the synthesis of proteins. *N*-glycans are instead synthesized in the complex biosynthetic pathway in which different enzymes and other proteins participate together with other molecules and factors in building of the glycan structure (Stanley et al. 2009; KEGG PATHWAY: *N*-Glycan biosynthesis) (detailed information on *N*-glycosylation pathway can be found in Chapter 1, *N*-glycosylation). Among main enzymes directly involved in synthesis of *N*-glycans are glycosyltransferases—the enzymes that add activated monosaccharides to the growing glycan. More than 200 genes that encode glycosyltransferases have been identified in humans (Carbohydrate Active Enzymes

Database 2020; Lombard et al. 2013), at least 40 of which have been assigned to protein *N*-glycosylation pathway (GGDB; KEGG PATHWAY: *N*-Glycan biosynthesis). Glycosyltransferases generally add monosaccharides in a specific order during *N*-glycan synthesis (Fig. 7.1) which means that a certain glycosyltransferase cannot act on the growing glycan if the glycan has not been modified in previous step(s) by some other specific glycosyltransferases. It is interesting to note that individual reactions of *N*-glycans synthesis that take place in the endoplasmic reticulum (ER) as well as first several reactions that occur in the Golgi apparatus are controlled by unique genes, meaning that there is no other gene encoding an alternative enzyme that can catalyze the same reaction (Fig. 7.1) (Breitling and Aebi 2013; KEGG PATHWAY: *N*-Glycan biosynthesis). On the other hand, for many further glycosylation reactions that take place in the Golgi, the same reaction may be catalyzed by two or more glycosyltransferases that are encoded by two or more different genes. Such reactions that can be catalyzed by more than one enzyme include those involved in elongation of *N*-acetylglucosamine (GlcNAc) branches (e.g., addition of galactose), additional branching, capping (e.g., addition of sialic acid), or antennary fucosylation of *N*-glycans (Fig. 7.1) (KEGG PATHWAY: N-Glycan biosynthesis). Also, it is known that glycosyltransferases that catalyze different reactions and are encoded by different genes can compete for the same acceptor glycan (e.g., glycosyltransferases encoded by *MGAT3*, *MGAT4A/B*, and *MGAT5/B* genes) (Abbott et al. 2008). Figure 7.1 provides an overview of genes encoding glycosyltransferases involved in the synthesis of *N*-glycans found on the human plasma proteins (Saldova et al. 2014; Zaytseva et al. 2020). However, although usually not found on human plasma proteins, some other proteins can contain *N*-glycans with the GlcNAc branches elongated with *N*-acetylgalactosamine (GalNAc) instead of galactose, or for example, sulfated *N*-glycans (Stanley and Cummings 2017). Thus, in addition to the glycosyltransferase genes shown in Fig. 7.1, protein products of some additional glycosyltransferase genes can be involved in formation of the final composition and structure of the *N*-glycan attached to a specific protein. For example, *GalNAc-T* genes that encode enzymes responsible for the addition of GalNAc residue and *CHST* genes that encode enzymes responsible for the addition of sulfate group to *N*-glycans as well as other genes (Stanley and Cummings 2017; GGDB).

Beside glycosyltransferase genes, products of many other glycogenes are involved in the *N*-glycan biosynthetic pathway. In particular, this concerns glycosidases which remove monosaccharides from the nascent glycan to create acceptor glycans for a certain glycosyltransferase. Other important classes of enzymes participating in *N*-glycosylation are those involved in synthesis of nucleotide-activated monosaccharides and their transport in the ER and Golgi (Stanley et al. 2009; Cummings and Pierce 2014; KEGG PATHWAY: *N*-Glycan biosynthesis).

It has been shown that the diversity and abundance of *N*-glycans present in specific cells or tissues are to some extent regulated at the level of the transcription of genes that code for proteins participating in glycan production and degradation (Nairn et al. 2008, 2012; Moremen et al. 2012). In addition to transcriptional regulation, it has also been suggested that the glycosylation process is regulated at a the post-transcriptional level where activity or localization of enzymes, competition between different glycosyltransferases, accessibility of the glycosylation site and other parameters may be important in determining the glycosylation status of a cell or tissue (Kukuruzinska and Lennon 1998; Nairn et al. 2008, 2012; Moremen et al. 2012).

7.3 Large Cohort Studies of Human Protein *N*-Glycosylation

Development of methods allowing high-throughput measurements of glycome in thousands of samples has opened a way to a data-driven investigation of biology of human

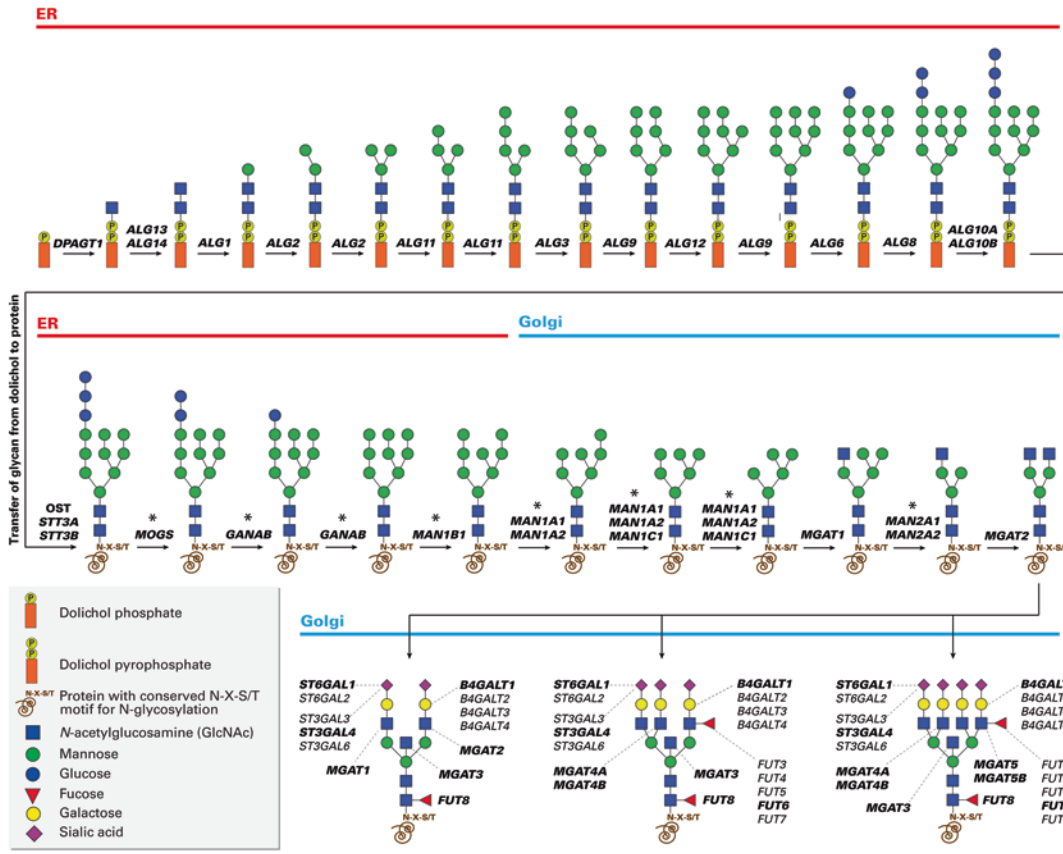


Fig. 7.1 Overview of genes involved in *N*-glycosylation of human plasma proteins. The initial steps of *N*-glycan synthesis take place in the endoplasmic reticulum (ER). Glycosyltransferases encoded by the *DPAGT1, ALG1-ALG3*, *ALG6*, *ALG8-ALG14* genes are involved in the synthesis of dolichol-linked glycan precursor. In addition, there are the *ALG5* and *DMP1-3* genes (not shown in the figure) which encode glycotransferases responsible for synthesis of dolichol-activated monosaccharides dolichol-P-glucose and dolichol-P-mannose, respectively. The latter serve as donor substrates for glycosyltransferases encoded by the *ALG3*, *ALG6*, *ALG8-ALG10A/B*, and *ALG12* genes. Transfer of glycan precursor from dolichol to a protein is catalyzed by the oligosaccharyltransferase (OST). OST is a protein complex composed of several proteins, and the *STT3A* and *STT3B* genes encode two isoforms of the catalytic subunit of the OST protein complex. The glycan precursor is transferred to the nitrogen atom of the asparagine residue within the conserved *N*-glycosylation sequence motif N-X-S/T (N = asparagine, X = any amino acid except proline, S = serine, T = threonine) of the target protein. After transfer to the protein, the trimming of glucose and mannose residues from *N*-linked glycans occurs. The *MOGS* and *GANAB* genes encode glucosidases—enzymes that remove galactose residue from the glycan, whereas the *MAN1B1* gene encodes mannosidase—an enzyme that removes mannose residue from the glycan. Genes encoding glycosidases are labeled with an asterisk (*) to make them distinguishable from genes encoding glycosyltransferases. After processing in ER, the protein with *N*-linked glycan is transferred from ER to Golgi, where further trimming of mannose residues by mannosidases (encoded by the *MAN1A1*, *MAN1A2*, and *MAN1C1* genes) takes place. In the Golgi, the formation of complex *N*-glycans is initiated by the sequential action of the enzymes encoded by the *MGAT1*, *MAN2A1/A2*, and *MGAT2* genes, respectively. The *FUT8* gene encodes a glycosyltransferase responsible for core fucosylation of *N*-glycans. The *MGAT3* gene encodes a glycosyltransferase required for the addition of a bisecting *N*-acetylglucosamine (GlcNAc). Additional GlcNAc branch formation (i.e., the formation of the third and fourth branches) is catalyzed by glycosyltransferases encoded by the *MGAT4A/B* and *MGAT5/B* genes. Elongation of GlcNAc branches by the addition of glucose residues is catalyzed by glycosyltransferases encoded by the *B4GALT1-4* genes. Capping of *N*-glycans with alpha-2-6-linked sialic acid is catalyzed by glycosyltransferases encoded by the *ST6GAL1* and *ST6GAL2* genes, while capping with alpha-2-3-linked sialic acid is catalyzed by enzymes encoded by the *ST3GAL3*, *ST3GAL4*, and *ST3GAL6* genes. The *FUT3-7* genes encode glycosyltransferases that add antennary fucose to *N*-glycans. The genes encoding the enzymes that, according to the literature data, are most responsible for catalyzing a specific reaction are indicated in bold. The literature consulted in preparing this figure (Stanley et al. 2009; KEGG PATHWAY: *N*-Glycan biosynthesis; GGDB; UniProt; Lee et al. 2001; Brinkman-Van der Linden et al. 1996; Chung et al. 2015; Mondal et al. 2015)

protein *N*-glycosylation. By the end of 2000s, the first large cohorts of thousands of individuals were profiled. It should be noted that the traits that are measured in high-throughput experiments are "glycan peaks" as accessed by one of the analytical chemistry techniques such as liquid chromatography, mass-spectrometry, or capillary gel-electrophoresis (Huffman et al. 2014). An area under such a peak is proportional to the abundance of glycans having similar physico-chemical characteristics. The abundance of most glycan peaks obtained using modern high-throughput techniques is determined by the presence of a single major glycan, although some peaks may contain two or more glycans with similar abundances (see Fig. 7.2). In that, although actual quantitative traits that are studied are the "glycan peaks," knowing that the majority of peaks are almost entirely determined by abundance of a specific single glycan allows for a meaningful interpretation. Moreover, this allows us to compute biochemically meaningful "derived traits" by adding up abundances of all peaks containing glycans that share certain properties. An example of such derived traits would be a proportion of core fucosylated structures, or a proportion of structures having a bisecting GlcNAc. In what follows we will speak about specific glycans and not about glycan peaks.

The importance of experimental design, raw data processing, and statistical quality control in large data-driven studies of *N*-glycosylation cannot be underestimated. An experimental design that fails avoiding confounding by experimental factors may generate spurious associations between glycans measured in an experiment and the characteristics of interest. A classical "extreme" example would be analyzing *N*-glycome of disease case samples and control samples in two separate batches. In this case an effect of the batch cannot be separated from the case/control status, and no valid conclusion can be drawn about association between *N*-glycans

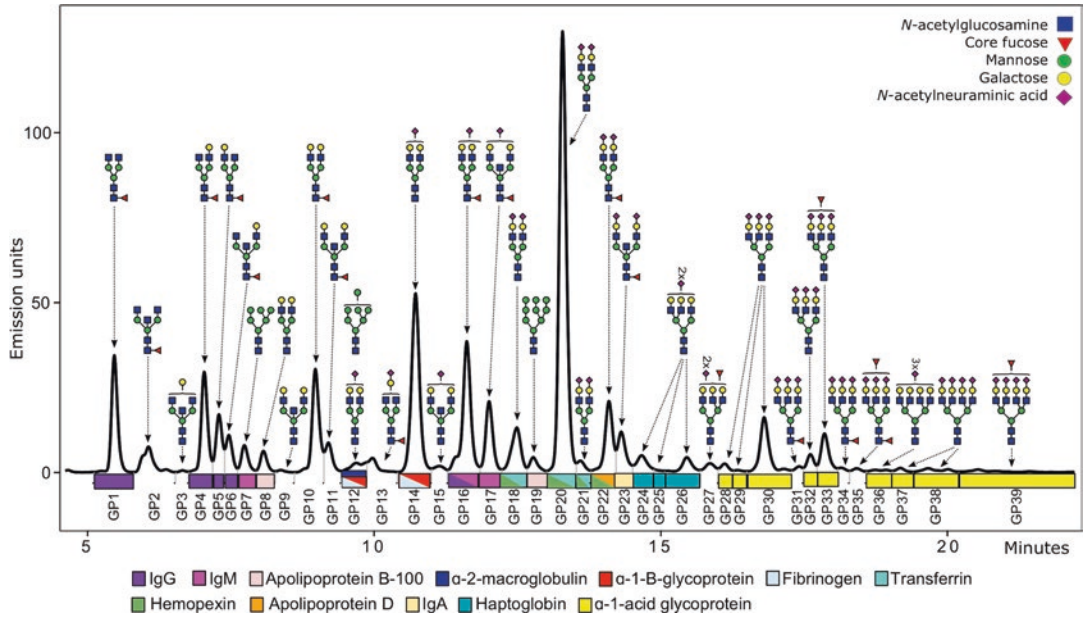


Fig. 7.2 Representative chromatogram of total human plasma *N*-glycans separated by ultra-high-performance liquid chromatography (UHPLC) into 39 glycan peaks (GP1 – GP39). Shown are structures of major *N*-glycans identified in each peak with liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. Color of the boxes under chromatographic peaks represents main glycoprotein sources of major glycan structures contained in the peak according to (Clerc et al. 2016). Reprinted with permission from (Zaytseva et al. 2020). Copyright 2020 American Chemical Society

and the disease. Even the most sophisticated statistical analysis will not be able to mend such inadequate experimental design. Although this example is somewhat artificial and is not expected to happen in real life, more subtle problems in design and raw data processing may be common. In addition to careful experimental design, it is also essential to ensure proper raw data processing, to model the data using distributions that are able to capture the features of the data and to perform a strict statistical quality control. Following these guidelines will increase power and could even mend some minor lapses in experimental design. On the contrary, a failure to ensure correct experimental design, improper raw data processing, and statistical quality control would lead to a loss of power, and to an increased rate of false-positives.

When designing an experiment for glycomic analysis, the basic principles of experimental design should be employed, namely blocking, randomization, and replication (Ugrina et al. 2017). It has been shown that glycosylation changes with age (Knezevic et al. 2010; Krištić et al. 2014) and that abundances of glycans are dependent on sex (Knezevic et al. 2010). Given that information about age and sex are almost always available, it is recommended that the experimental design should, whenever possible, be blocked by age and sex (Ugrina et al. 2017). This means that when, due to large number of samples, samples from the same collection need to be analyzed on multiple plates, then samples should be randomly assigned to plates while at the same time ensuring that the age distribution and sex ratio on each plate are the same as within the entire original collection of samples. Furthermore, depending on the specifics of the study, some other blocking variables may be used, for example, in case-control studies, the experimental design should, in addition to age and sex, also be blocked by case/control status (Ugrina et al. 2017).

The current glycome data processing often assumes total area normalization of glycome profiles followed by a log-transformation, batch effect correction (Goh et al. 2017), and derived traits estimation. Recently it was proposed that the probabilistic median quotient normalization may have better properties (Uh et al. 2020; Benedetti et al. 2020), and this normalization approach has started gaining popularity (Sharapov et al. 2020; Freidin et al. 2016). Among different batch-correction algorithms, ComBat (Johnson et al. 2006) remains the most popular. Computations of the derived traits is among the latest steps that finish glycome data processing. Commonly used derived traits, such as abundance of glycans with bisected GlcNAc, abundance of core-fucosylated glycans, etc., are hoped to provide a proxy to the enzymatic activities of glycan synthesis enzymes.

Up until now, large cohort studies investigating human *N*-glycosylation were mostly limited to an investigation of *N*-glycosylation of plasma proteome and/or *N*-glycosylation of one of the most abundant plasma glycoproteins, immunoglobulin G (IgG). Below we will attempt to summarize results from genetic studies of both plasma proteome and IgG glycomes, and will make a distinction between the two only when it is necessary.

Already very early epidemiologic studies have demonstrated that levels of the majority of glycans are significantly associated with such demographic factors as sex and age. For some glycan peaks, the correlation with age was as strong as 0.5 (Knezevic et al. 2009); jointly, age and sex explained a large (up to 33%) proportion of total variance of some glycan peaks (Pučić et al. 2011). Especially the effects of age are very pronounced for some glycans: a GlycanAge index composed of the three IgG glycan peaks was shown to explain about 60% of variance of chronologic age (Krištić et al. 2014). These observations led to a common practice that stated that essentially in any statistical analysis including glycans the effects of sex and age should be taken into account. In all analyses discussed in this chapter glycans were considered as dependent variables. Adjustment for sex and age is done either by fitting the effects of sex and age together with other independent variables, or, in case when orthogonality of effects could be assumed, adjustment is done by studying the glycan residuals.

7.4 Heritability of Human Plasma Protein *N*-Glycosylation

A narrow-sense heritability (h^2) is defined as a proportion of the trait's variance that could be attributed to additive genetic effects. The higher is heritability, the higher is genetically determined correlation between the phenotypes of relatives. Taking an extreme scenario, for a hypothetical trait with heritability of 100%, two twins, even when raised apart, would be identical; for a hypothetical trait with heritability of 0%, two twins raised apart would be expected to be as different as two random people from the population. One of the most popular tools that allows estimation of heritability of quantitative traits is a study of correlations between phenotypes of monozygotic and dizygotic twins. The same design allows partitioning the variance of quantitative traits into components due to additive genetic variation, and effects from shared and unique environments. A meta-analysis of 17,804 traits from 2748 publications demonstrated that across all human traits studied, an "average" reported heritability is 49% (Polderman et al. 2015). It has also been demonstrated that estimates of heritability cluster strongly within functional domains. For a majority (69%) of human traits, the observed twin correlations are consistent with a simple and parsimonious model where twin resemblance is solely due to additive genetic variation. No substantial influences from a shared environment or non-additive genetic variation were detected for a majority of traits.

Early family-based studies of plasma *N*-glycome estimated an average heritability of the 29 glycan peaks as 35%, with SD = 16% and range from 1% to 58% (Knezevic et al. 2009). These early studies used high performance liquid chromatography (HPLC) to access *N*-glycome. The drawback of this procedure was relatively high experimental error and relatively low resolution, that is, some glycan peaks contained a mixture of glycans. The first would clearly reduce heritability, while the second does not allow obtaining an objective picture of distribution of heritabilities across specific glycans. Another peculiarity of the work of Knezevic and colleagues was that heritability was estimated using a large extended pedigree including 1008 measured individuals from a genetically isolated population. While this approach has many advantages, unlike the twin design, it does not allow estimation of the effects of a shared environment in a simple and straightforward manner.

A recent work by Zaytseva and colleagues (Zaytseva et al. 2020) has overcame many of the above limitations by using an up to date ultra-high-performance liquid chromatography (UHPLC) to measure 39 glycan peaks in blood plasma samples collected from 269 MZ and 772 DZ twin pairs. This allowed using classical genetic epidemiologic methods to estimate the contribution from additive genetic, common and unique environments into the variation of 39 glycan peaks. An average heritability reported for 39 glycan peaks was 48%, with SD = 18% and a range from 17% to 74%. In general, more abundant glycan peaks have a lower experimental error of measurement. An experimental error increases the unique environmental variance. Hence it may be expected, on average, that more abundant glycan peaks may have somewhat higher heritability. Indeed, authors concluded that more abundant glycan peaks tended to have higher heritability, although the two most abundant peaks exhibited low heritability, while one of the least abundant peaks showed high heritability.

The highly heritable peaks contained core-fucosylated biantennary *N*-glycans with low levels of sialylation. Such glycans are mainly present on immunoglobulins. Other peaks with high heritability mostly contained di- and trisialylated species with two or three antennae. These glycans are expected to derive from such glycoproteins as haptoglobin, transferrin, IgA, apolipoprotein D, alpha-1-glycoprotein, and others. Majority of the 15 peaks with low heritability contained glycan species found on various glycoproteins produced by the liver.

For the vast majority of plasma *N*-glycans, the best model for variance partitioning was the one assuming additive genetic effects and unique environment as the major sources of variation.

The contribution from the familial environment shared between the twins to plasma *N*-glycan variability was demonstrated for two traits only, leading authors to conclude that the contribution from the shared environment is very limited. Similar overall results were obtained in studies of heritability of the 23 IgG glycan peaks (Pučić et al. 2011; Menni et al. 2013).

To conclude, the variation of *N*-glycan abundances is determined jointly by genetic and environmental factors. The range of heritabilities observed for *N*-glycans is similar to that of complex human traits (Polderman et al. 2015). Heritabilities of glycans also seem to exhibit other features common to complex human traits, such as clustering of estimates of heritability within functional domains and the fact that for a majority of traits, the observed twin correlations are consistent with a simple and parsimonious model where twin resemblance is solely due to additive genetic variation.

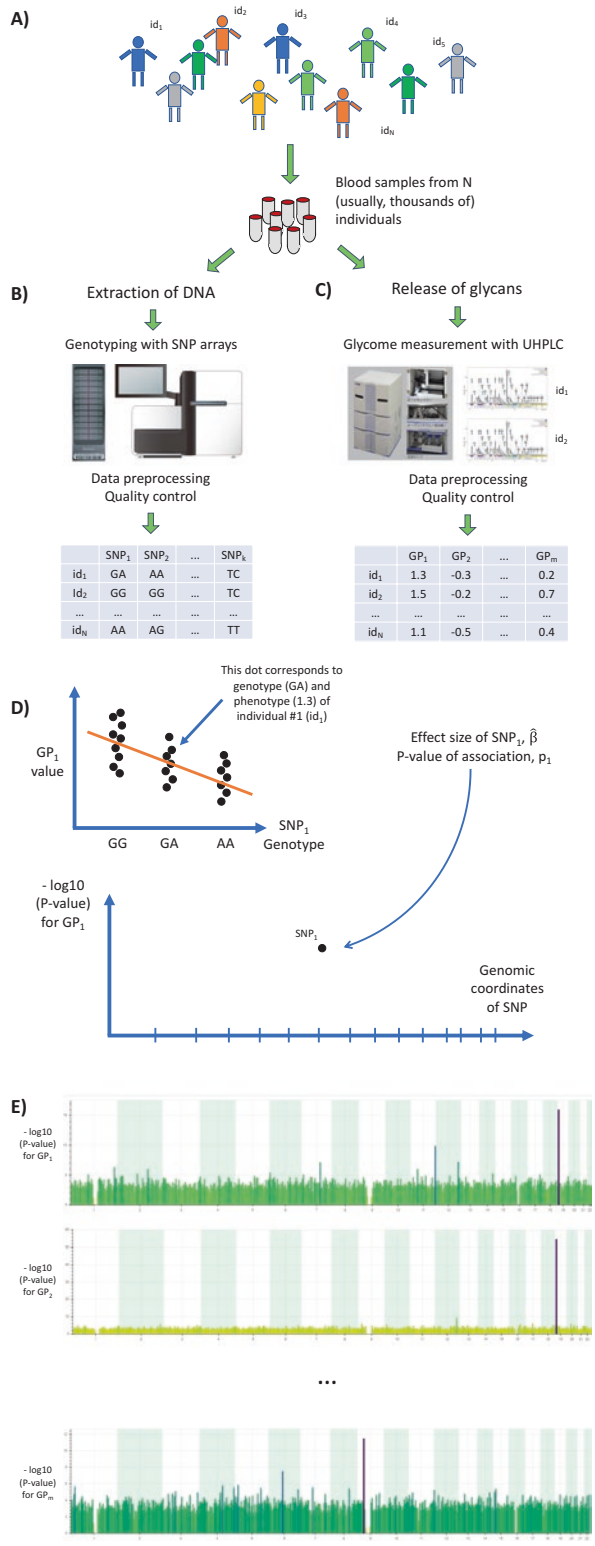
Heritability studies allow estimation of the proportion of trait variability that is determined by additive genetic variation. However, these studies do not answer the question where in the genome this genetic variation is located, and which specific DNA changes lead to changes in the value of the trait. The mapping of determinants of *N*-glycan variation to the genome can be done by GWAS, that we consider in the next section.

7.5 Genome-Wide Association Studies of Human Plasma Protein *N*-Glycosylation

The progress in high-throughput *N*-glycome profiling methods allowing characterization of thousands of samples, together with availability of DNA technologies allowing affordable and precise measurements of common variation, has set a scene for application of GWAS to understand genetic determinants of variation of human glycome. GWAS has a potential to discover (possibly subtle) effects of common genetic variation, e.g., SNPs, onto abundances of glycan structures by comparing genomes and *N*-glycomes of thousands of individuals (Fig. 7.3).

In a GWAS one typically investigates correlation between genotypes of each of the millions of SNPs distributed throughout the genome with the glycan quantitative traits. While the SNPs measured in a GWAS do not necessarily include all functional variants that affect *N*-glycosylation, the presence of linkage disequilibrium (LD)—that is, correlation between genotypes of physically proximal SNPs—ensures that effect of any common polymorphism will likely be detectable, given sufficient balance between the SNP's effect size and the size of the sample. Millions of statistical tests are done in a GWAS, and a stringent statistical significance threshold has to be used when making claims about significant association. In studies of genome-wide association of a single complex trait with common (minor allele frequency > 1%) polymorphisms, a threshold of $p < 5 \cdot 10^{-8}$ is used to claim genome-wide significant association. In studies of *N*-glycome, further correction for multiple testing needs to be done, as many directly measured and derived quantitative traits are studied. In this case, a common practice is to perform an additional Bonferroni correction for the "effective" number of traits, which, essentially, is the number of eigenvectors that capture the majority (>99%) of the total variance. In case of IgG glycome, that is often characterized with 77 traits, the effective number is 21, leading to the genome-wide significance threshold of $5 \cdot 10^{-8}/21 = 2.4 \cdot 10^{-9}$ (Klarić et al. 2020); in case of plasma glycome including 113 traits, the effective number is 29, leading to the threshold of $1.7 \cdot 10^{-9}$ (Sharapov et al. 2019). While this stringent multiple testing correction may feel almost excessive, this approach leads to highly robust results and has worked well to the benefit and credibility of the field. A recent well-powered study of (Sharapov et al. 2020) demonstrated that for the vast majority (at least 15 out of 16) of plasma *N*-glycome loci claimed as genome-wide significant using the above-described procedure the effects could be reliably reproduced in a set of independent samples.

The gold-standard GWAS design involves identification of the loci that are associated at the genome-wide significance level with a trait of interest in a discovery set and subsequent replication of the identified associations in an indepen-



	SNP1	SNP2	...	SNPk
id1	GA	AA	...	TC
Id2	GG	GG	...	TC
...	...	...	...	...
idN	AA	AG	...	TT

	GP1	GP2	...	GPm
id1	1.3	-0.3	...	0.2
id2	1.5	-0.2	...	0.7
...	...	...	...	...
idN	1.1	-0.5	...	0.4

Fig. 7.3 Idealized glycomics GWAS workflow. (**a**) The thousands of participants of a study are recruited and the blood is collected. The blood is used to extract DNA and plasma. (**b**) The DNA is genotyped using, e.g., SNP arrays. The genotypic data undergo quality control and imputations. At the end, the data can be presented as large

dent set (Bush and Moore 2012). Replication improves the reliability of GWAS findings and helps to reduce the chance that the observed genotype–phenotype association is a chance finding or an analysis artifact (Kraft et al. 2009). Replication is, therefore, an essential step to be taken before complex and expensive follow-up studies are initiated.

Identification of loci associated with glycome is assumed to provide starting points to understand better the glycobiology. This, however, is not trivial as neither functional polymorphism, nor the affected gene follows immediately from a GWAS. A locus, identified in a GWAS, could encompass dozens of genes; moreover, an unknown functional element in the locus may regulate a gene that is located far beyond the associated region (Smemo et al. 2014). We will discuss the challenges of linking an association signal to a biological function in the next section.

As for the moment, we will turn to a review of GWAS of human *N*-glycome. In discussion of identified loci, we will often tag them with a name of a candidate gene. These candidate genes are either "obvious" candidates—such as glycosyltransferases—that are known from glycan biochemistry, or are the candidates that were prioritized in a laborious process of in silico or experimental functional follow-up.

To date, three GWAS of human plasma *N*-glycome have been published (Lauc et al. 2010; Huffman et al. 2011; Sharapov et al. 2019). In the first two studies performed by (Lauc et al. 2010) and (Huffman et al. 2011), *N*-glycans were measured using HPLC technology. A pilot study of GWAS by (Lauc et al. 2010) analyzed 13 glycan traits in 2705 individuals and discovered three genome-wide significant loci, one of which included the *HNF1A* gene and others included genes encoding fucosyltransferases *FUT6* and *FUT8*. In 2011, (Huffman et al. 2011) extended the analysis to 46 glycan traits. From these 33 were directly measured and 13 were derived and averaged glycosylation features such as branching, galactosylation, sialylation, and other features across different individual glycan structures. They also increased the sample size to 3533 individuals. These refinements led to the identification of three additional loci (Fig. 7.4). However, none of these studies used independent samples to replicate their findings. Finally, the recent study by (Sharapov et al. 2019) analyzed a total of 113 glycan traits, of which 36 were directly measured by UHPLC—a method more advanced and accurate than HPLC—and 77 were derived traits. Examining data on 2763 individuals, (Sharapov et al. 2019) replicated five loci discovered in previous works (all except *SLC9A9*) and identified 10 new loci. Seven out of these 10 new loci were replicated in an independent multicenter cohort of 1048 samples. At that stage, 16 loci were implicated at genome-wide significance level, with 12 of them replicated in independent samples.

Sharapov and colleagues (Sharapov et al. 2020) summarized and verified the plasma glycome GWAS efforts by using the largest set of samples so far (4802 individuals) to replicate previously identified loci. For all but one locus, the expected replication power exceeded 95%. Of the 16 loci reported previously, 15 were replicated in that study. For the remaining locus (near the *KREMEN1* gene), the replication power was low,

Fig. 7.3 (continued) tables with thousands of rows and millions of columns. In the example, genetic information from each participant is presented as a row, and each column contains genotype probabilities of a specific SNP across all participants. (**c**) The glycans are released from plasma proteins and chromatographic spectra are obtained. The data undergo multiple steps of quality control, pre- and post-processing, including computation of derived traits. The results can be presented as data tables with thousands of rows and tens of columns. In the example, each row corresponds to a single sample, and columns correspond to glycan traits. (**d**) An elementary unit of GWAS analysis is a regression of the values of a glycan trait (in the example, GP_1) on the genotype. The p-value for a test against the null hypothesis of no association between the trait and the genotype is computed. The base-10 logarithm of 1/p is then located on the Y-axis of a Manhattan plot, that has the genomic coordinate of the SNP on the X-axis. (**e**) Repeating step (D) for association of GP_1 with every SNP provides a GWAS for GP_1. The procedure is repeated for GP_2 and so forth, generating a series of GWAS, one for each investigated trait. Copyright (2020) S. Sharapov, Y. Aulchenko; distributed under a Creative Commons Attribution License 4.0 (CC BY)

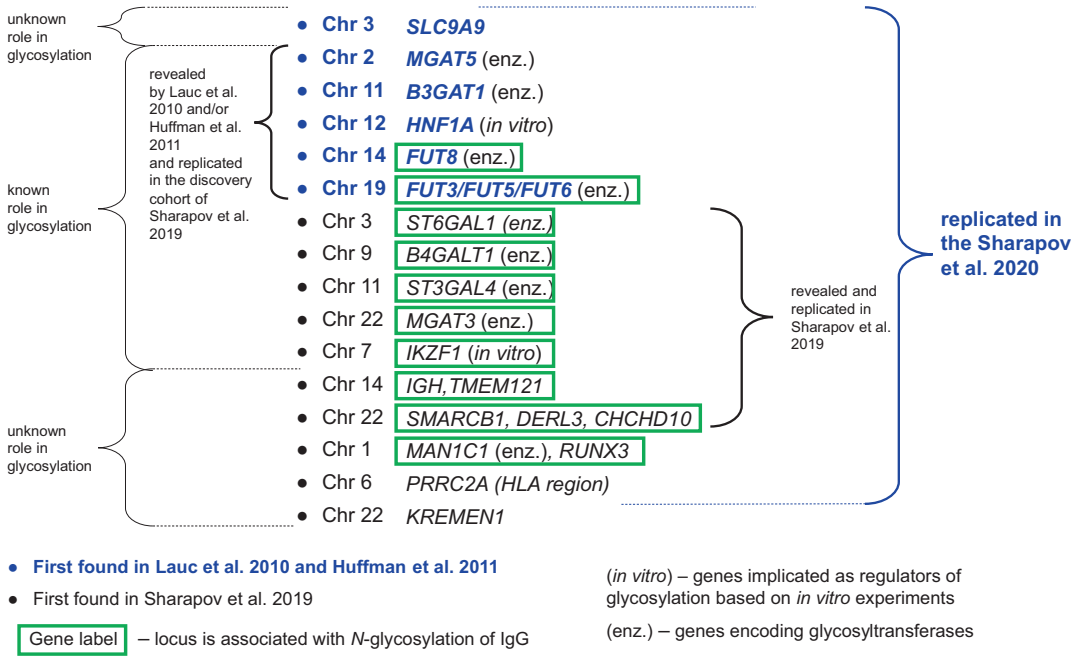


Fig. 7.4 Schematic overview of the known plasma protein *N*-glycome-associated loci. Chr—chromosome. Green boxes highlight loci associated with *N*-glycosylation of IgG as reported by (Lauc et al. 2013; Shen et al. 2017; Klarić et al. 2020; Wahl et al. 2018). The (enz.) label highlights genes that encode enzymes with known roles in glycan biosynthesis. The (in vitro) label highlights genes whose regulatory role in the glycosylation process was shown experimentally (Lauc et al. 2010; Klarić et al. 2020). Adopted from (Sharapov et al. 2020), by permission of Oxford University Press

and hence, replication results were inconclusive. The very high replication rate highlights the general robustness of the GWAS findings as well as the high standards adopted by the community that studies genetic regulation of protein glycosylation. The 15 replicated loci present a good target for further functional studies. Among replicated loci, eight contain genes encoding glycosyltransferases: *MGAT3*, *MGAT5*, *B3GAT1*, *FUT8*, *FUT6*, *ST6GAL1*, *B4GALT1*, *ST3GAL4*. The remaining seven loci offer starting points for further functional follow-up investigation into molecules and mechanisms that regulate human protein *N*-glycosylation.

Thus, 16 loci associated with plasma *N*-glycome have been identified to date, and 15 of them have been replicated (Fig. 7.4).

As for the IgG-glycosylation, four GWASs were reported to date (Lauc et al. 2013; Shen et al. 2017; Wahl et al. 2018; Klarić et al. 2020). The first GWAS of IgG glycome identified associated variants in or near 4 glycosyltransferase genes and in 5 additional loci with no apparent role in protein glycosylation (Lauc et al. 2013). The list was extended by six additional loci (Shen et al. 2017; Wahl et al. 2018), only one of which contained a glycosyltransferase gene. While these studies were limited to discovery-stage sample size of about 2000 individuals, a work of (Klarić et al. 2020) almost quadrupled this sample size by investigating associations between IgG glycome and genome in a discovery sample of about 8000 individuals. The results were replicated in 2400 samples with UHPLC-measured IgG glycome and validated in about 1800 samples where IgG glycome was measured using liquid chromatography-mass spectrometry (LC-MS). Overall, this work brought the number of loci that were genome-wide significantly associated with IgG glycome to 27, of which 22 were replicated in independent samples. Additionally, to the loci that con-

tain glycosyltransferase genes (*MGAT3*, *ST6GAL1*, *B4GALT1*, *FUT3/FUT5/FUT6*, *FUT8*), previous analyses identified loci that contain genes encoding transcription factors known to play central roles in lymphocyte maturation and differentiation (*RUNX1*, *RUNX3*, *IKZF1*, and *IKZF3*). One interested in details of these loci is referred to Table 1 of Klaric et al. (Klarić et al. 2020).

7.6 Inferences from Genetic Studies of Human Protein *N*-Glycosylation

While replication establishes a locus as a solid candidate for further investigation, neither discovery GWAS, nor replication does per se answer the question of the biological function of a locus. On one hand, presence of LD in human genome is very beneficial in that we do not need to deeply sequence whole genomes of study participants to investigate associations genome-wide, but can instead genotype only the "tagging SNPs" that capture information from the majority of common polymorphisms via LD. At the same time, presence of LD limits the precision of mapping of a functional polymorphism (see Fig. 7.5). In that, GWAS allows mapping functional determinants of complex traits to loci that encompass large regions including tens and even hundreds of kilobase pairs. In terms of the list of potentially functional polymorphisms, each of these loci may include tens or even hundreds of candidate polymorphisms. Only extremely large and powerful studies are able to map associations to just a few SNPs per locus (Huang et al. 2017). To complicate the story even further, a gene affected by a functional element perturbed by a SNP may be located megabases apart from the signal of association (Smemo et al. 2014).

Identification of causal polymorphisms and genes that explain association is critical in translating statistical findings of GWAS into actionable biological knowledge. Given that in every locus a number of polymorphisms, genes, and mechanisms are present to choose from, a variety of potential mechanistic explanations of an association could be huge. Functional follow-up in a laboratory is expensive and time-consuming. Therefore, relatively cheap and fast in silico functional investigation, that allows prioritizing possible mechanisms has gained extreme popularity in post-GWAS studies. Such in silico methods exploit a large body of knowledge accumulated on the potential consequences of changes in DNA (McLaren et al. 2016; Rogers et al. 2017), functional role of different genomic elements (Moore et al. 2020; Kundaje et al. 2015), and data accumulated in omics studies (Pers et al. 2015), including these investigating genetic control of omics traits (GTEx Consortium et al. 2017; Momozawa et al. 2018; Sun et al. 2018; Shin et al. 2014). The latter data are especially useful when dissecting potential molecular genetic mechanisms underlying specific associations. Methods of colocalization analysis (Giambartolomei et al. 2014; Zhu et al. 2016; Momozawa et al. 2018) are used to address the question if association of two traits to the same genetic region may be due to pleiotropic effects of the same genetic variant(s), or, rather, is a consequence of association of the two traits to two distinct, although located close by, polymorphisms.

Here, we will summarize in silico as well as in vitro functional studies that were motivated by human glycome GWAS, and will discuss a number of loci in detail. To start with, it is interesting to analyze an overlap between results generated by plasma and IgG glycome GWAS (see Fig. 7.4, loci enclosed in green boxes). A large overlap is expected, not only because of possible similarities between genetic regulation of *N*-glycosylation between tissues, but also because one the most abundant glycoproteins of plasma is the IgG (Clerc et al. 2016). In that, one may expect to eventually see all loci associated with IgG-glycosylation in a very powerful plasma glycosylation GWAS, but not other way around.

Here we will quickly review loci that appear to be plasma glycome-specific. One of these is a locus enclosing the *MGAT5* gene. The *MGAT5* codes for the enzyme mannosyl (α-1,6-)-glycoprotein β-1,6-*N*-acetyl-glucosaminyltransferase V (GnT-V), which adds GlcNAc residues

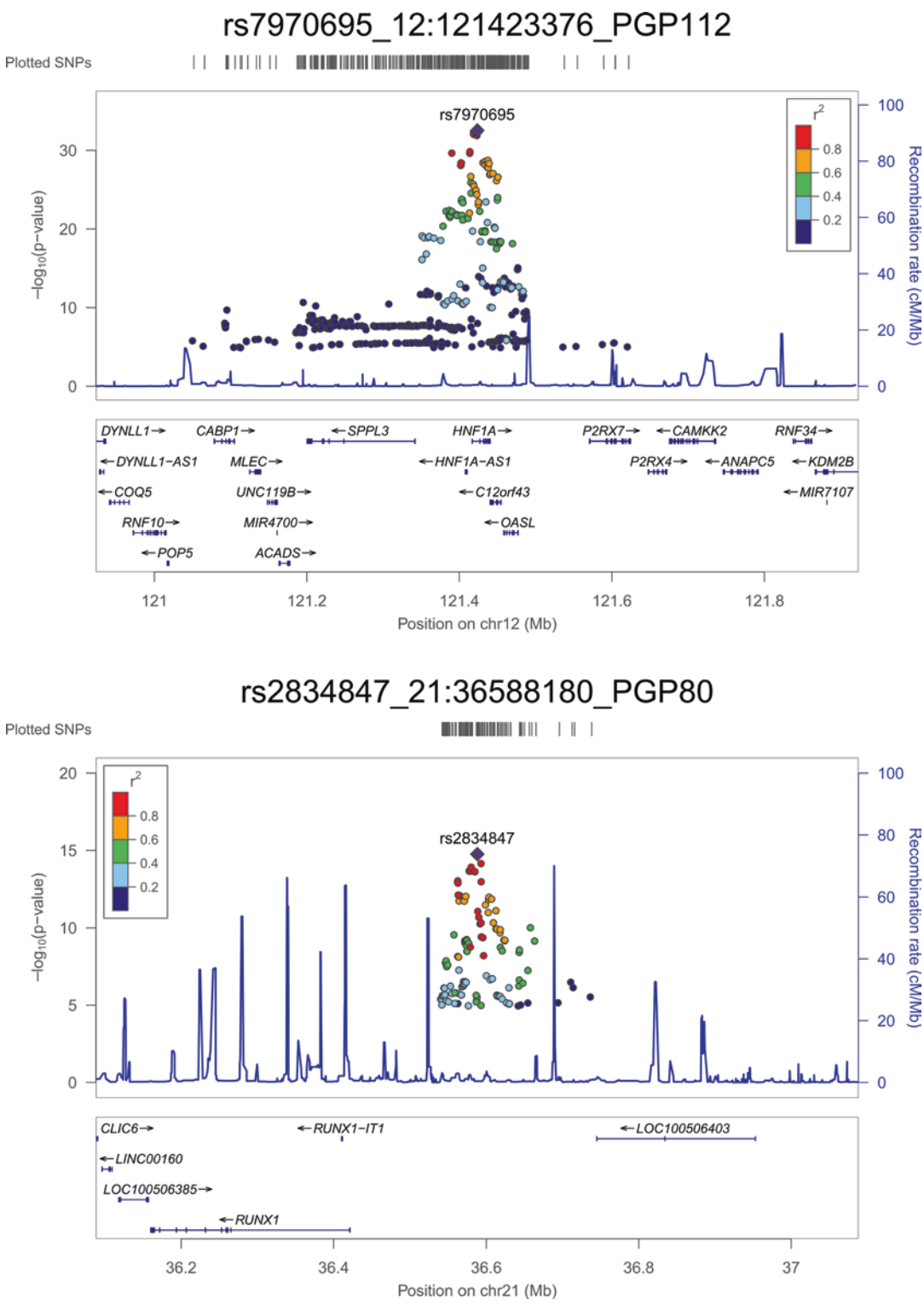


Fig. 7.5 Examples of regional association plots visualizing association between a trait and polymorphisms located in specific locus. A region of association can include multiple genes (**a**) or no genes (**b**). Association p-values>10^{-5} not shown

to mannose in a β1,6 orientation on the antennary structure of *N*-glycans. This is a decisive step in the generation of tetra-antennary glycans, and indeed, the variation in abundance of tetra-antennary structures was associated with this region (Lauc et al. 2013; Sharapov et al. 2020). Such structures are not attached to IgG and their origin is, most likely, from liver proteins. In the locus near *HNF1A*, the *HNF1A* itself is considered an established candidate that was confirmed by experimental studies. Lauc et al. (2010) have shown that *HNF1A* and its downstream target *HNF4A* regulate the expression of key fucosyltransferase and fucose biosynthesis genes and demonstrated that *HNF1A* is both necessary and sufficient to drive the expression of these genes in hepatic cells. Thus plasma-specific loci near *MGAT5* and *HNF1A* are likely affecting glycosylation of plasma glycoproteins produced in the liver via established mechanisms.

The situation with the plasma-specific locus near *B3GAT1* is less clear. Although this locus is associated with variation in abundance of tetra-antennary structures, which indicates that its action is not through glycosylation of immunoglobulins, the mechanism of action of this locus is not obvious. The role of *B3GAT1* as a candidate gene is reinforced by colocalization analysis that suggests that the same functional variant may explain association with plasma glycan levels as well as expression of *B3GAT1* in blood, lung (Sharapov et al. 2019), and liver (our unpublished results). *B3GAT1* is a member of the glucuronyltransferase gene family, encoding glucuronyl transferase that acts in the biosynthesis of the carbohydrate epitope HNK-1, adding a glucuronic acid (GlcA) to the terminal *N*-acetyllactosamine (LacNAc) disaccharide to form the HNK-1 epitope precursor (Oka et al. 1992; Mitsumoto et al. 2000). The HNK-1 epitope is expressed on a subset of human lymphocytes, including natural killer cells. However, this epitope was not reported to exist on plasma proteins. Huffman et al. (Huffman et al. 2011) performed mass spectrometry (MS) analysis of the DG13 plasma glycan peak that was associated with *B3GAT1* region in their work. They demonstrated the presence of glucuronic acid on some glycans contained in this peak.

The locus near *SLC9A9* (solute carrier family 9 member A9) does not contain any gene that is an obvious candidate for involvement in glycosylation. Authors who discovered and confirmed association to this locus (Huffman et al. 2011; Sharapov et al. 2020) suggested that *SLC9A9* itself may be a plausible candidate. This gene encodes a sodium/proton exchanger which is suggestively involved in the regulation of endosomal pH (Roxrud et al. 2009), and glycosylation is likely to be highly sensitive to changes in Golgi luminal pH (Kellokumpu 2019). Based on analysis of literature, Sharapov and colleagues (Sharapov et al. 2020) speculate that alterations in *SLC9A9* function might affect sialylation of bisected (addition of bisected GlcNAc) glycans via modulation of Golgi pH.

Now we will turn to inferences from analysis of IgG-glycome GWAS. The latest work of Klaric et al. (Klarić et al. 2020) performed extensive in silico functional investigation. Exploiting the multidimensional nature of glycomic data, the authors have built a network connecting associated loci (Fig. 7.6). In this network, the closely located nodes are those having similar spectrum of glycans associated to them.

The presence of glycosyltransferases in the network allows for functional inferences via an interesting approach. Namely, similarity of the spectrum of glycans affected by some locus with the spectrum associated with an action of a well-characterized enzyme suggests that the locus contains a candidate that in some way regulates the activity of the enzyme. In particular, it follows that loci containing *IKZF1*, *IKZF3* may regulate the *FUT8*, an enzyme, responsible for core-fucosylation of IgG. Indeed, an experimental study demonstrated that the knockdown of *IKZF1* in a lymphoblastoid cell line leads to decreased expression of *IKZF3*, increased expression of *FUT8* and increased core-fucosylation of IgG (Klarić et al. 2020), confirming suggested sub-network.

Interestingly, colocalization analysis of the *ORMDL3-GSDMB-IKZF3* locus suggested that the same pleiotropic functional variant may be

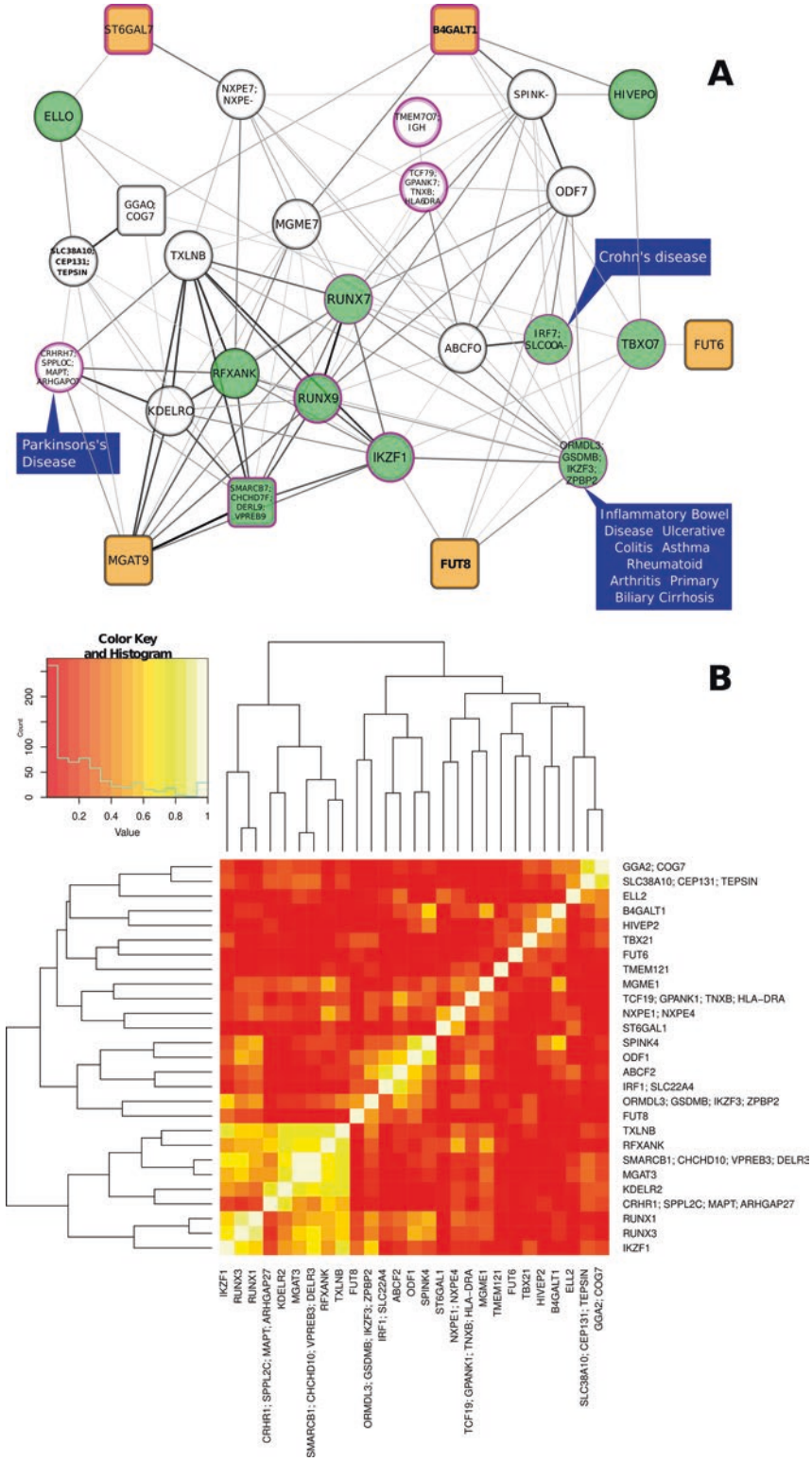


Fig. 7.6 (**a**) Functional network of loci associated with IgG *N*-glycosylation. In this network, each node represents a lead SNP in the locus, and each edge represents the squared correlation of glycome-wide effects of the two nodes. Only correlations that were significant after multiple testing correction ($p \leq 1.4 \times 10^{-4}$) are shown. The

responsible for increased expression of *IKZF3*, decreased expression of *ORMDL3* and *GSDMB*, decreased fucosylation and increased proportion of agalactosylated afucosylated IgG glycans, and the risk of ulcerative colitis and rheumatoid arthritis (Klarić et al. 2020). Core-fucosylation of IgG is known to act as a safety switch preventing antibody-dependent cellular cytotoxicity (ADCC) (Shields et al. 2002), while agalactosylated afucosylated structures are considered to be proinflammatory (Karsten et al. 2012). Taking all this into account, we may speculate that, perhaps, the mechanism of action of the *ORMDL3-GSDMB-IKZF3* locus onto the risk of a range of autoimmune and inflammatory diseases is, at least in part, mediated by the interplay been *IKZF1*, *IKZF3*, and *FUT8* and the effects this interplay exhibit on the *N*-glycome of IgG. Colocalization analysis also suggested possible pleiotropy between variants affecting glycans and the risk of Crohn's disease at the locus containing *IRF7* gene and Parkinson disease at the locus containing *MAPT* gene (Fig. 7.6). Further computational and experimental functional studies are needed to detail, confirm, or refute this hypothesis.

7.7 Conclusions and Perspectives

Although changes in glycome are observed in a wide range of diseases and pathological states, the examples of use of glycans as biomarkers and therapeutic targets is limited. This is not in small part because the understanding of human glycome regulation is incomplete and fragmented. Although biochemical reactions involved in glycosylation are well understood, the mechanisms regulating these reactions in cell-, tissue-, and environment-specific manner are virtually unknown.

Combination of human glycomics and genomics offers a powerful "data-driven hypotheses" approach to dissect the complex human glycobiology in vivo in an agnostic manner. The GWASs of human *N*-glycome start revealing regulators of the biochemical network of *N*-glycosylation. Some of these regulators seems to exhibit pleiotropic effects on human disease, especially autoimmune and inflammatory.

In the future, we expect that more powerful GWAS will detect further loci that are pleiotropic between glycans and human diseases and provide more material to systematically investigate and understand the mechanisms that link glycans with human health and disease.

At the moment, large-scale human cohort studies are limited to investigation of total blood plasma protein and IgG glycomes. Widening the range of tissues and proteins would allow more detailed, precise, and targeted investigation of glycome. However, as tissue samples are hardly available, especially from healthy individuals, single-protein glycomic studies are likely to first focus on using plasma/serum as an easily accessible sample source for isolation and subsequent analysis of glycosylation of individual proteins other than just IgG. Glycosylated proteins present in plasma at high to medium abundance include, in addition to IgG, transferrin, fibrinogen, IgA, alpha-2-macroglobulin, IgM, alpha-1-antitrypsin, complement proteins, haptoglobin, apolipoproteins, and alpha-1-acid glycoprotein (Anderson and Anderson 2002). Since these glycoproteins originate from one of two sources—either from the liver or from antibody producing cells—simply by studying different plasma proteins we may be able to gain a better understanding of the specifics of glycosylation in different tissues/cell types.

Fig. 7.6 (continued) edges are thicker if correlation is stronger. Round-edged rectangular nodes denote genes that are, according to gene ontology (GO), involved in glycosylation; purple-edged nodes denote genes involved in immune system processes; green nodes denote loci containing genes involved in transcription regulation; orange nodes denote glycosyltransferases; and blue rectangles indicate diseases pleiotropic with IgG glycans in the given locus. (**b**) Hierarchical clustering of pairwise Spearman's locus-effect correlations. Reproduced from (Klarić et al. 2020). Copyright (2020) The Authors; distributed under a Creative Commons Attribution License 4.0 (CC BY)

Dissection of the glycome into its single-protein components will be complemented by multi-omics studies of other omics domains of the relevant tissues. Most importantly, this will be the transcriptome. Taking these data together, aligning genomics with glycomics with other omics will allow deep study of interplay of genes and molecular phenotypes. Understanding this interplay will provide a rich source of etiologic insight, candidate therapeutic interventions, and biomarkers.

The hypotheses generated in this data-driven manner will need to be targeted in functional wet-lab experiments. Such functional follow-up experiments should employ a combination of in vitro and in vivo model systems to provide evidence that genes and mechanisms suggested by computational methods are indeed involved in regulation of protein glycosylation.

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Disclosure of Interests YSA is a cofounder of PolyOmica and PolyKnomics BV. JK is an employee of Genos Ltd. The rest of the authors declare no potential conflict of interest.

Ethical Approval This work is a literature review and, as such, does not contain studies with human participants or animals performed by any of the authors.

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8 Epigenetic Regulation of Glycosylation

Rossella Indellicato and Marco Trinchera

Abstract

Expression of glycosylation-related genes (or glycogenes) is strictly regulated by transcription factors and epigenetic processes, both in normal and in pathological conditions. In fact, glycosylation is an essential mechanism through which proteins and lipids are modified to perform a variety of biological events, to adapt to environment, and to interact with microorganisms.

Many glycogenes with a role in normal development are epigenetically regulated. Essential studies were performed in the brain, where expression of glycogenes like *MGAT5B*, *B4GALNT1*, and *ST8Sia1* are under the control of histone modifications, and in the immune system, where expression of *FUT7* is regulated by both DNA methylation and histone modifications. At present, epigenetic regulation of glycosylation is still poorly described under physiological conditions, since the majority of the studies were focused on cancer. In fact, virtually all types of cancers display aberrant glycosylation, because of both genetic and epigenetic modifications on glycogenes. This is also true for many other diseases, such as inflammatory bowel disease, diabetes, systemic lupus erythematosus, IgA nephropathy, multiple sclerosis, and cardiovascular diseases.

A deeper knowledge in epigenetic regulation of glycogenes is essential, since research in this field could be helpful in finding novel and personalized therapeutics.

Keywords

Cancer · Epigenetics · Glycosylation · Glycogenes · Gene expression · Inflammatory bowel disease (IBD)

8.1 Introduction

Epigenetics and glycosylation are two essential mechanisms occurring every second in our organism. To understand the importance of them, it is necessary to start from the foundation: the genome. The genome is the template of an organism and gives rise to an elaborate network of interacting biological molecules. Every organism interacts with the surrounding environment and every cell of a multicellular organism is interconnected with the other cells. The adaptation to the environment and the communication between

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cells is given by the network of biological molecules (thus only indirectly by the genome), which regulate epigenetic and glycosylation processes: the first is able to control when, where, and how a coding sequence of DNA will be transcribed, the latter has the capacity to alter protein and lipid structure and function (Lauc et al. 2014).

Another layer of complexity is added when epigenetics and glycosylation cross their path, in a sort of circular scheme, and the result is both epigenetic regulation of glycosylation and glycosylation as a way to regulate epigenetics. In this chapter, only epigenetic regulation of glycosylation will be discussed.

The gold standard definition of epigenetics was formulated in 2008 at a Cold Spring Harbor meeting and establishes that "an epigenetic trait has been defined as a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al. 2009). The DNA sequence is generally stable, and it is the same in every cell of an organism, yet cells have not all the same phenotype. In fact, they are able to differentiate into many different types, to perform several functions, and to adapt and respond to the environment. Epigenetics is the mechanism that allows all this to happen. Basically, epigenetic processes are essential to orchestrate gene expression of a cell and organ development (Jaenisch and Bird 2003).

Before starting to describe the mechanisms through which epigenetics operates, it is necessary a specification: the term "epigenetics" is often used as a synonym for "epigenetic inheritance," but even if these two processes use the same mechanisms to attend to their functions, they are not the same. In fact, epigenetic regulation of gene expression is a process that acts during the differentiation of somatic cells, as well as in response to environmental changes (Lind and Spagopoulou 2018), whereas epigenetic inheritance permits the stable transmission of epigenetic changes to the next generation of individuals through germ cells (Skvortsova et al. 2018).

Epigenetics works mainly through DNA methylation, histone modifications (acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, ribosylation, citrullination, and *O*-GlcNAcylation) and non-coding RNAs (especially microRNAs and long non-coding RNAs) (Greville et al. 2016; Bannister and Kouzarides 2011; Cavalli and Heard 2019; Tsai and Yu 2014; Zoldos et al. 2012).

DNA methylation in vertebrates occurs in CpG dinucleotides. The CpG dinucleotides tend to cluster in regions called CpG islands (CpG-rich regions), which are found in about 60% of human gene promoters. They are usually unmethylated in normal cells, even though they can be methylated in a tissue specific manner during development, in differentiated tissues (Portela and Esteller 2010) or even abnormally methylated during carcinogenesis. The mechanism of DNA methylation is quite simple: a methyl group (CH_3) is covalently attached to the 5-carbon of the cytosine residue (5mC) in these CpG sites with the aid of DNA methyltransferases (DNMTs) (Skvortsova et al. 2018; Greville et al. 2016; Dawson and Kouzarides 2012) and normally this process is associated with gene silencing (Portela and Esteller 2010). Upon DNA methylation, gene silencing occurs by two mechanisms: (1) the methyl group physically interrupts the binding of transcription factors to their recognition sequences, and (2) methyl-CpG binding proteins bind to the methylated DNA and recruit co-repressor molecules, including histone deacetylase, to induce chromatin structure condensation (Oda et al. 2013).

As far as concern histone modifications (primarily acetylation and methylation), they can either activate or repress gene transcription through alterations in the chromatin structure (Greville et al. 2016). Chromatin can be divided into actively transcribed euchromatin and transcriptionally inactive heterochromatin (Portela and Esteller 2010). Generally, euchromatin is characterized by high levels of acetylation and di/tri methylation of H3K4, H3K36, and H3K79 (Portela and Esteller 2010; Norouzitallab et al. 2019), whereas heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27, and H4K20 methylation (Portela and Esteller 2010).

Finally, non-coding RNAs belong to several classes, for example microRNAs (miRNAs) and

long non-coding RNAs (lncRNAs) among others. miRNAs (20–30 nucleotides in length) regulate post-transcriptional processes (Cavalli and Heard 2019) and are involved in gene silencing (Dawson and Kouzarides 2012), in fact they target mRNA 3′UTR regions and inhibit protein translation or enhance mRNA degradation (Greville et al. 2016). LncRNAs are more variable in length (up to more than 100 kilobases) (Cavalli and Heard 2019) and can act as molecular chaperones or scaffolds for various chromatin regulators (Dawson and Kouzarides 2012).

If you have arrived at this point of the book, you should already know that glycosylation is the most abundant and complex modification in mammals that alter lipid and protein structure. It frequently takes place in a cell- and tissue-specific manner, since it is essential to maintain various physiological functions in multicellular organs (Lauc et al. 2014; Moremen et al. 2012; Kizuka et al. 2014). It is noteworthy to underline that, contrary to proteins and DNA which are linear molecules, glycans are complex branched structures that are defined not only by the sequence of monomeric units, but also by the exact position of the glycosidic bond, its anomeric configuration (α or β), the number of branches, the position of branching (Lauc and Zoldos 2010). The final glycan structure is also given by environmental factors, genetic factors (i.e., single nucleotide polymorphisms – SNPs), transcription factors, protein transports, altered pH in Golgi, Golgi organizers, ion channels, oxygen concentration, subcellular localization of enzymes, activated monosaccharide donor substrates, and glycan acceptor substrates availability (Lauc et al. 2014; Klasic et al. 2016; Nairn et al. 2008). Over 800 glycogenes (glycosyltransferases, glycosidases, and enzymes for sugar nucleotide biosynthesis and transport among others) are thus required for protein and lipid glycosylation (Taniguchi et al. 2014). Since glycosylation is cell- and tissue-specific, the fine-tuning of all these glycogenes is entrusted not only at transcriptional level by transcription factors, but mostly by epigenetic information (Lauc et al. 2014). Epigenetic regulation of glycosylation is gaining more importance in these last two decades because it generates the diversity that higher eukaryotes require to assemble complex structures, adapt to environment and interact with microorganisms (Lauc et al. 2014). Unluckily, the detailed mechanism of the tissue-specific epigenetic regulation of glycogenes both in health and disease is still poorly understood (Kizuka et al. 2014, 2016).

8.2 Epigenetic Regulation of Glycosylation, Environment, and Evolution

Epigenetics provides the link between environment, genotype, and phenotype (Fig. 8.1) (Jaenisch and Bird 2003; Ladd-Acosta and Fallin 2016). Following an environmental change, epigenetics can alter the activity of glycogenes and thus the composition of biological structures, favoring adaptation to specific environmental conditions (Zoldos et al. 2012; Tangvoranuntakul et al. 2003). This adaptation is (relatively) stable and so, even if the environmental factors (such as diet, exposure to pharmacological agents, different kinds of stress) are not present any longer, their prolonged effect can be preserved through epigenetic cell memory (Lauc and Zoldos 2009; Zoldos et al. 2010, 2013a).

An accurate example of this environment-phenotype connection is related to the alteration of homeostasis, during acute systemic inflammation, in which composition of both total plasma and immunoglobulin G (IgG) glycome can change rapidly. Upon restoration of homeostasis, the glycome composition returns to the characteristic initial state of an individual (Klasic et al. 2016). Also psychological stress can be a powerful environmental factor, so that it is able to change the glycan structure in the human gastric mucosa and plasma (Lauc and Zoldos 2010).

All these epigenetic changes are always accomplished so that an organism is more fit for a specific environment. If these changes are transmitted to a germ cell and then to the next generation of an individual through the so-called epigenetic inheritance, the epigenetic regulation of glycosylation can be considered an essential

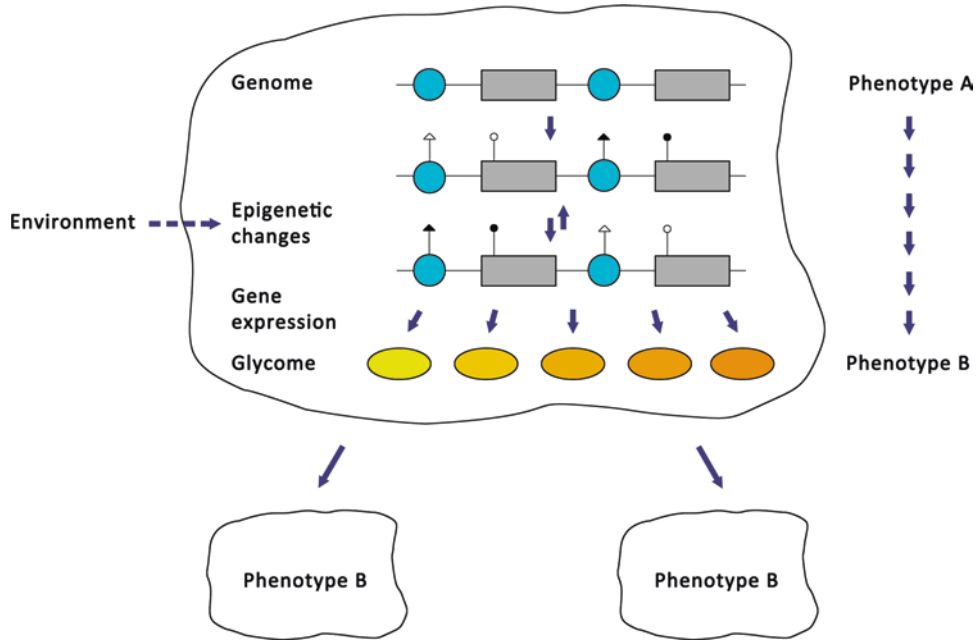

Fig. 8.1 Representative scheme of genome, epigenome, and glycome network. Environment plays a pivotal role in the epigenetic changes of a cell, which will modify its phenotype accordingly. Depending on which glycogenes are affected by epigenetic changes, different kind of glycans will arise. Daughter cells will acquire the new epigenetic asset and phenotype. Light blue circle: histone; gray rectangle: gene; white dot: unmethylated CpG site; black dot: methylated CpG sites; white triangles: active mark on histone; black triangles: repressive mark on histone

evolutionary mechanism, enabling quick adaptation of complex organisms to environmental conditions (Zoldos et al. 2013b). This is particularly true when it comes to competition with pathogens. In fact, pathogenic and commensal microorganisms bind to our cells through glycan receptors (Varki and Gagneux 2017). Human population, over time, developed resistance mechanisms against specific pathogens and the hypothesis presented by Lauc and colleagues (Lauc et al. 2014; Lauc and Zoldos 2009) is that this resistance resulted from a modification in the gene expression patterns, leading to adaptive glycosylation in the immune system and to variability in the membrane glycoconjugates, which were passed to the next generations by epigenetic inheritance. Stable epigenetic alteration can create and maintain novel structural features in higher organisms without introducing mutations in the DNA, that is very risky for complex organisms with a limited number of offspring. As a consequence, exploiting epigenetic marks, higher eukaryotes are capable to outmaneuver rapidly evolving pathogens. A well-fitting example is given by the Sda antigen, in fact bacterial adhesins may be specific for α2,3-sialylated glycoconjugates, but in the Sda antigen there is the β1,4-linked *N*-acetylgalactosamine (GalNAc) residue that hinders the attachment of the pathogenic bacteria expressing these adhesins. In fact, the localization of the Sda antigen in tissues and organs, such as colon and kidney, in close contact with the external environment is consistent with this hypothesis (Dall'Olio et al. 2014).

8.3 Epigenetic Regulation of Glycosylation in Health

It has been proven that human plasma glycome presents high variability among individuals (up to even 50% for some glycan structures)

(Knezevic et al. 2009), but it carries a great temporal stability within an individual, even if, after a period of approximately 1 year, greater differences in the glycan profile were observed (Gornik et al. 2009). This general stability can be addressed to either genetic polymorphisms or by stable epigenetic differences in transcriptional status of glycogenes (Zoldos et al. 2010). Epigenetic regulation of glycosylation is the mechanism which can explain both the temporal stability of the glycome in healthy individuals as well as specific changes which were reported to appear during different physiological processes (i.e., development and aging of an organism) or in various diseases (Zoldos et al. 2010; Horvat et al. 2013).

It is time to go deeper in the studies on epigenetic modifications involved in protein and lipid glycosylation. In fact, as it has been said before, many glycogenes with a role in normal development are epigenetically regulated. This was proven for example by doing experiments on cell cultures: using epigenetic inhibitors, the *N*-glycome profiles or composition of neural glycolipids drastically change, and this was an evidence that many glycogenes are regulated both by DNA methylation and histone modification (Lauc et al. 2014; Kizuka et al. 2014).

8.3.1 Brain

A deeper knowledge of how glycans are regulated in the brain is essential, since neural cells such as neurons and astrocytes are known to express unique functional glycans not found in other cells or tissues, such as human natural killer-1 (HNK-1), polysialic acid, and branched *O*-mannose glycans. These glycans play critical roles in high-order brain functions (i.e., learning/memory, formation of neural networks, or myelination) and various neurological disorders. However, little is known about how the spatial and temporal expression of these neural glycans is established and maintained (Kizuka et al. 2016).

The most prominent works in this field were performed by two groups and concern both glycogenes involved in *N*- and *O*-glycosylation and in lipid glycosylation.

8.3.1.1 GnT-IX (*N*-Acetylglucosaminyltransferase IX)

The human gene encoding GnT-IX is *MGAT5B*. It catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to the 6-OH position of the mannose residue of GlcNAcβ1,2-Manα on both the α1,3- and α1,6-linked mannose arms in the core structure of *N*-glycan, but it is also responsible for the transfer of GlcNAc in β1,6-linkage to *O*-mannosyl glycan and it is exclusively expressed in the brain (Inamori et al. 2004).

Neural GnT-IX expression is under the control of neural cell-specific histone modifications, as it was evidenced by Kizuka and colleagues (Kizuka et al. 2011). They performed experiments on both cell cultures and mice tissues. As far as concerns cell cultures, they used 3T3-L1 cells (derived from murine adipose tissue, that shows no expression of GnT-IX mRNA) and Neuro2A (mouse neuroblastoma cells, that show expression of GnT-IX mRNA) and observed that using Trichostatine A (TSA), a HDAC inhibitor, on 3T3-L1 cells, there was a marked increase of acetylation on histone 3 lysine 9 (H3K9ac), which corresponds to a typical active chromatin mark, and a consequent strong induction of GnT-IX transcription. Also, examining histone code-based chromatin activation state around the *GnT-IX* core promoter and the transcription start site (TSS) region by chromatin immunoprecipitation (ChIP) analysis, it was observed that the *GnT-IX* TSS region of 3T3-L1 cells was associated with repressive chromatin marks, such as H3K27me3 and H3K9me2, while the same region of Neuro2A cells was associated with active chromatin marks, such as H3K9ac and H3K4me3. The same happens in mice tissues: in mouse brain the *GnT-IX* TSS region is associated with active chromatin mark, whereas liver and kidney with repressive chromatin mark. Moreover, expression of other neural glycosyltransferases (GlcAT-P and ST8Sia-IV) is likely to be regulated by a similar epigenetic mechanism (Kizuka et al. 2011).

More recently, the same group found out how chromatin around glycosyltransferase promoters is activated in specific tissues. The chromatin modifiers that are involved in epigenetic regulation of the mouse *GnT-IX* gene are an epigenetic suppressor, HDAC11, and an activator, the OGT-TET3 complex. The first works in *GnT-IX* negative cells, while in contrast OGT-TET3 complex is a prerequisite for the efficient binding of the transactivator, NeuroD1, to the *GnT-IX* promoter to drive it (Kizuka et al. 2014). It is worth of notice to underline that *OGT* (*O*-GlcNAc transferase) is a glycogene that just recently was added to the growing list of epigenetic modification (Hanover et al. 2012).

8.3.1.2 B4GALNT1 and ST8Sia1

It is not possible to talk about brain without mentioning the gangliosides. They are a class of sialic acid-containing glycosphingolipids, particularly abundant in the central nervous system. The quantity and the expression pattern of gangliosides in brain is ontogenically regulated and they are mainly regulated through stage-specific expression of ganglioside synthase genes. On this purpose, epigenetic regulation of two key glycosyltransferases, that is, *B4galnt1* (GM2/GD2 synthase) and *St8sia1* (GD3 synthase), was investigated in embryonic, post-natal, and adult mouse brains. Brain gangliosides shift from the simpler ones (GM3 and GD3) in early phases of life to more complex ones during development (GM1, GD1a, GT1a, and GT1b). *B4galnt1*, rather than *St8sia1*, is more closely associated with conversion of simpler gangliosides into more complex ganglioside, in fact the mRNA expression of B4galnt1 was drastically increased during development, whereas the St8sia1 expression level revealed only a slight increase. Successively, by ChIP assay, histone acetylation levels of the two genes were analyzed. It emerged that the histone H3 acetylation levels drastically increased in the *B4galnt1* gene and slightly increased in the *St8sia1* gene during development, consistently with the mRNA expression pattern. To better confirm this study, neuroepithelial cells (NECs) from mouse embryos, which are rich in neural stem cells (NCSs), were treated with a histone deacetylase inhibitor, sodium butyrate. As a result, expression of GM3 ganglioside decreased, while expression of complex gangliosides (GD1a, GD1b, and GT1b) increased, suggesting that efficient histone acetylation of the glycosyltransferase genes in mouse brain contributes to the developmental alteration of ganglioside expression (Suzuki et al. 2011).

A few years later, the same group elucidated the differential regulatory mechanisms underlying epigenetic activation of the same two glycogenes, adding that not only H3 acetylation, but also H4 acetylation (AcH3/AcH4) of the gene's 5′-flanking region in chromatin is correlated with developmental expression pattern of *B4galnt1* and *St8sia1* genes. AcH3/AcH4 on the *B4galnt1* promoter leads to recruitment of trans-activation factors Sp1 and AP-2. Experiments on NCSs mouse culture showed that only with double knock-down of HDAC1 and HDAC2, the expression of *B4galnt1* mRNA is up-regulated and Sp1 and AP-2 loading is significantly increased, reflecting the level of histone acetylation. On the contrary, levels of mRNA, binding of transcription factor, and status of acetylation histone remained unchanged for *St8sia1* gene. This indicates that transcription of *B4galnt1* and *St8sia1* can be regulated by different HDAC isoforms because double knock-down of HDAC1 and HDAC2 leads to *B4galnt1* gene trans-activation, but not *St8sia1* (Tsai and Yu 2014; Itokazu et al. 2018).

8.3.2 FUT7 (α-1,3-Fucosyltransferase, FucT-VII)

FUT7 is involved in the formation of sialyl Lewis X (sLex) antigen, the carbohydrate ligand for E and P selectins (Taniguchi et al. 2014), that is expressed in leukocytes and $CD4^+$ T effector cells, but not in naive T cells, and it is responsible for the trafficking of those cells into inflamed areas (Syrbe et al. 2004). In 2004, Syrbe and colleagues found out that FUT7 expression after activation of naive T cells is dependent on progression through the cell cycle. Most importantly, they demonstrated that DNA methylation is

involved in the expression of this glycogene, since by treating $CD4^+$ T cells with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR), the expression of selectin ligand increased. This effect of DNA demethylation strongly suggest that induction of selectin ligands is epigenetically modulated in lymphocytes during differentiation (Syrbe et al. 2004).

Another important regulation of FUT7 in T helper lymphocytes subpopulations (Th1 and Th2) is given by histone acetylation, in fact FUT7 and sLex are preferentially expressed in Th1 cells. The mechanism involves two transcription factors, T-bet and GATA3. T-bet is a Th1 cell-specific transcription factor, whereas GATA3 is a Th2 cell-specific transcription factor. GATA3 is able to recruit histone deacetylase (HDAC)-3 and − 5 and thus, to suppress FUT7 transcription in Th2 cells. This was also proven by the addition of TSA in Jurkat cells, in which transcription of FUT7 was enhanced (Chen et al. 2006).

A new insight into tissue-specific expression of FUT7 was achieved by Pink and colleagues (Pink et al. 2014). Experiments on mice CD4+ T cells and lung fibroblasts were performed and it was proven that the active histone modification H3K4me2 mark was scattered in all analyzed T cell populations across the *Fut7* gene, whereas lung fibroblasts, which lack Fut7 mRNA and selectin ligand expression, carried a differential histone modification pattern with an enrichment of repressive H3K4me3 mark and absence of active H3K4me2 mark. This suggests that the *Fut7* locus is accessible but that other mechanisms (such as transcription factors) control the inducible expression in CD4+ cells and that the pattern of histone modifications might control tissue-specific expression of *Fut7*.

8.4 Epigenetic Regulation of Glycosylation in Cancer

Literature on physiological regulation of glycosylation through epigenetics is not so extensive and it is still under investigation. Instead, a broad number of reports have focused on epigenetic mechanisms affecting glycosylation in cancer (reviewed in ref. (Dall'Olio and Trinchera 2017) and in Table 8.1), since it is well known that one of the hallmark of cancer is aberrant glycosylation.

Many studies are based on investigation of the methylation status of the promoter region of the glycogene, usually through the use of demethylating agents, such as 5-aza-CdR, even if reactivation of a potential hypermethylated promoter is not always established, since other histone modification marks are involved (Dall'Olio et al. 2012).

It was also shown that epigenetic regulation of glycosyltransferases in cancer cells leads to the creation of novel glycan structures, that can be one of the mechanisms used by cancer cells to evade the host immune response (Lauc and Zoldos 2009; Kannagi et al. 2008; Kawamura et al. 2008; Kim and Deng 2008).

8.4.1 B4GALNT2 (β-1,4-*N*-Acetyl-Galactosaminyltransferase 2)

B4GALNT2 is an enzyme involved in the synthesis of the Sda antigen, which is a histo-blood group antigen that contributes to the definition of the individual immunophenotype. Sda antigen could be expressed on the surface of erythrocytes, in several tissue types, such as stomach, colon, kidney and oocytes, and in secretions (urine, saliva, milk, serum) (Dall'Olio et al. 2014; Wang et al. 2008), but more than half of the people whose red cells are Sda negative, still present Sda in the urine or saliva. This indicates that a different regulation of the biosynthesis of this antigen occurs at least in kidney, salivary glands and bone marrow (Dall'Olio et al. 2014). Very little is known about the normal regulation of expression of *B4GALNT2*, except that it has been suggested to be ontogenically regulated (i.e., the expression varies during development) after experiments on guinea-pigs (Dall'Olio et al. 1987) and rats (Dall'Olio et al. 1990), in which the enzyme was absent at birth and increased with age. However, the feces, urine, and saliva of human newborns have been reported to express high Sda activity (Morton et al. 1970) but it is

Table 8.1 List of the principal glycogenes regulated by epigenetic mechanisms

Target	Epigenetic mechanism	Effect	Tissue/cells involved	References
Galactosyltransferases				
B4GALT3	miR-1247-3p (up-regulated) CircUBXN7/miR-1247-3p axis	Down-regulation Up-regulation	CAFs in lung metastasis of liver cancer Bladder cancer	Fang et al. (2018) Liu et al. (2018a)
N-acetyl-galactosaminyl transferases				
GALNT1	LncRNA SNHG7/miR-216b axis	Up-regulation	Colorectal cancer	Shan et al. (2018)
GALNT3	Linc01296/miR-26a axis	Up-regulation	Colorectal cancer	Liu et al. (2018b)
GALNT4	miR-4262 (down-regulated)	Up-regulation	Colorectal cancer	Qu et al. (2017)
GALNT7	miR-30e (down-regulated) LncRNA SNHG7/miR-34a axis miR-154 (down-regulated) miR-125a-5p (down-regulated)	Up-regulation Up-regulation Up-regulation Up-regulation	Cervical cancer Colorectal cancer Laryngeal squamous cell carcinoma Cervical cancer	Wu et al. (2017) Li et al. (2018) Niu et al. (2018) Cao et al. (2020)
B4GALNT1	Histone acetylation Hypermethylation	Up-regulation Down-regulation	Renal cell carcinoma Hepatocellular carcinoma	Banerjee et al. (2019) Sun et al. (2018)
N-acetyl-glucosaminyl transferases				
OGT	miR-483 (down-regulated)	Up-regulation	Gastric cancer	Yu et al. (2018)
Sialyltransferases				
ST6GAL1	LncRNA ZFAS1/miR-150 axis LncRNA HOTAIR/miR-214 axis	Up-regulation Up-regulation	T-cell acute lymphoblastic leukemia Colorectal cancer	Liu et al. (2019a) Liu et al. (2019b)
ST6GAL2	LncRNA HCP5/miR-22-3p, miR-186-5p, miR-216a-5p axis	Up-regulation	Follicular thyroid carcinoma	Liang et al. (2018)
ST6GALNAC2	miR-182 and miR-135b	Down-regulation	Colorectal cancer	Jia et al. (2017)
ST6GALNAC3	Promoter hypermethylation	Down-regulation	Prostate cancer	Haldrup et al. (2018)
ST6GALNAC5	Promoter hypermethylation	Down-regulation	Cervical cancer	Verlaat et al. (2018)
ST6GALNAC6	Histone methylation	Down-regulation	Colon cancer	Huang et al. (2019)
ST8SIA1	Promoter hypomethylation	Up-regulation	Triple negative breast cancer	Li et al. (2019)
ST8SIA4	miR-146a and miR-146b (up-regulated)	Down-regulation	Follicular thyroid carcinoma	Ma et al. (2017)
Fucosyltransferases				
FUT1	miR-34a (down-regulated)	Up-regulation	Head and neck squamous cell carcinoma	Wang et al. (2017)
FUT4	miR-26a/b (down-regulated) miR-200b (down-regulated) miR-29b/Sp1 axis	Up-regulation Up-regulation Up-regulation	Colorectal cancer Breast cancer Acute myeloid leukemia	Li et al. (2017) Zheng et al. (2017) Liu et al. (2019c)
FUT5	miR-125a-3p (down-regulated)	Up-regulation	Colorectal cancer	Liang et al. (2017)
FUT6	miR-125a-3p (down-regulated) LncRNA HOTAIR/miR-326 axis	Up-regulation Up-regulation	Colorectal cancer Colorectal cancer	Liang et al. (2017) Pan et al. (2019)

still not clear the reason of this discrepancy. More information is available when it comes to the down-regulation of its expression in gastrointestinal cancers. In fact, in different human gastrointestinal cancer cell lines neither B4GALNT2 transcript nor Sda antigen has been found (Wang et al. 2008). Moreover, according to a different study (Kawamura et al. 2008), all gastric cancer tissues examined lacked Sda antigen expression. Since putative promoter regions of the human *B4GALNT2* gene are embedded in CpG island, it has been proven that DNA methylation plays a crucial role in the epigenetic regulation of *B4GALNT2* glycogene (Dall'Olio and Trinchera 2017). In fact, using the demethylating agent 5-aza-CdR (Kawamura et al. 2008; Wang et al. 2008), it has been observed a partial recovery of the *B4GALNT2* gene expression. On the contrary, when human colon cancer cells were also treated with butyrate (a histone acetylase inhibitor) (Kawamura et al. 2008), neither alterations in the Sda antigen nor *B4GALNT2* gene expression was observed. As proposed by Zoldos and colleagues (Zoldos et al. 2010), these results suggest that it is the DNA methylation rather than histone deacetylation that contributes to the down-regulation of *B4GALNT2*.

8.4.2 B3GALT5 (β1,3-Galactosyltransferase Isoenzyme 5)

B3GALT5 is the enzyme responsible for the synthesis of type 1 chain carbohydrate antigens, in particular it participates in the biosynthesis of Lewis a, Lewis b, and sialyl Lewis a. Expression of *B3GALT5* is driven by two main promoters: native promoter and LTR promoter. Native promoter is mainly active in mammary gland, thymus, and trachea, as well as in some human cancer cell lines, it is sensitive to nuclear factor NF-Y and it is epigenetically regulated through the methylation of two flanking CpG islands. LTR promoter is mainly active in the organs of the gastrointestinal tract (colon, stomach, and pancreas), it is sensitive to hepatocyte nuclear factor HNF1α/β, but no CpG island is present in the proximal sequence (Trinchera et al. 2014), leading to the hypothesis that some stretches of CG dinucleotides upstream and non-adjacent to the LTR promoter could act as potential epigenetic regulators of transcription (Aronica et al. 2017). In fact, it has been proven that NF-Y and HNF1 are necessary to activate respectively the native and the LTR promoters, but they are unable to modulate transcription, which depends on distal regulatory elements that need to be unmethylated for the native promoter or methylated for the LTR promoter. This is an intriguing example, since it highlights a new aspect of transcriptional control: not only DNA hypermethylation of CpG islands during carcinogenesis is responsible for the silencing of a gene, but also hypomethylation of distant sequences cooperate on one gene to obtain full cancer-associated silencing (Trinchera et al. 2014).

Transcriptional activity of native promoter is also associated to chromatin status, in fact high expression of the transcript was found to be associated with active histone marks (H3K4me3, H3K79me2, H3K9Ac, and H3K9-14Ac), while low levels of transcript are associated with repressive histone marks (H3K27me2 and H4K20me3) (Caretti et al. 2012).

Examples of this analysis can be found in the differential regulation of B3GALT5 in colon and pancreas.

As far as concern native promoter, in the pancreas very low levels of methylation were found in samples, without differences between normal and cancer tissues, whereas in matched normal and tumor colon samples, the methylation levels of both CpG islands were increased in cancer with respect to the corresponding normal mucosa, leading to a strong down-regulation of the transcript (Caretti et al. 2012).

As far as concern LTR promoter, in the pancreas expression levels of B3GALT5 LTR transcript were similar to, or even lower than, those of the native transcript, without difference between normal and cancer tissues for both transcripts (Aronica et al. 2017), whereas high levels of expression of LTR B3GALT5 transcript were found in the normal colon mucosa and really low levels were found in the tumor counterpart, due

to the demethylation of a distant DNA sequence (Caretti et al. 2012; Zulueta et al. 2014).

These data indicated that the methylation-dependent mechanism of silencing reported in colon cancer appears to be inactive in the pancreas (Aronica et al. 2017).

8.5 Epigenetic Regulation of Glycosylation in Other Diseases

Dysregulation of glycosylation is not only associated with cancer, but also with a wide range of other diseases, such as diabetes, inflammatory bowel disease, systemic lupus erythematosus, multiple sclerosis, cardiac diseases, autoimmune diseases, IgA1 nephropathy, and others. The literature is not as extensive as the one involving cancer, but still there are some prominent examples.

8.5.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) comprises two chronic intestinal inflammatory disorders, namely Crohn's disease (CD) and ulcerative colitis (UC) (Khor et al. 2011). As far as concerns epigenetic of glycosylation and its association with this disease, the variation on DNA methylation was analyzed in relation to susceptibility to IBD. Differential methylation was analyzed in rectal biopsies from patients with CD and UC (both inflamed and non-inflamed tissues) and from healthy controls, using methylation microarrays. Several glycogenes were found to be hyper- or hypomethylated in inflamed UC vs controls, inflamed CD vs control, and non-inflamed UC vs controls, whereas there was no significant differential methylation between non-inflamed CD vs controls. In inflamed UC vs controls, B3GALT2, GFPT1, and GBGT1 have increased methylation; in inflamed CD vs controls, GFPT1 and GBGT1 have increased methylation and FUT2 has a decreased methylation; in non-inflamed CD vs controls, FUT7 and FGF23 have decreased methylation. These differential methylations (together with many others) correlated with the development of IBD, pointing out the contribution of these specific genes to IBD pathogenesis (Cooke et al. 2012).

Promoter methylation of *MGAT3* was investigated by pyrosequencing assay in DNA from whole blood and from separated $CD19^+$ B cells, from PBMCs, from $CD3^+$ T cells isolated from PBMCs, and from $CD3^+$ T cells isolated from the colonic mucosa of healthy controls (HC) and UC patients.

CpG sites within the *MGAT3* promoter were hypermethylated in disease compared to healthy individuals both in whole blood and $CD19^+$ B cells. The same pattern of CpG methylation differences was observed in PBMCs of the IBD patients and HC. A correlation analysis was performed between the *MGAT3* promoter methylation and individual IgG glycans. Increase in *MGAT3* promoter methylation correlated with a decrease in certain galactosylated and sialylated structures. In addition to the decreased levels of bisecting GlcNAc on non-galactosylated glycans, the most significant effect of the *MGAT3* promoter methylation on IgG glycome composition was a decrease of IgG galactosylation. Acquired pro-inflammatory properties of IgG antibodies mediated by alteration in IgG Fc glycosylation suggests that *MGAT3* gene plays an important role in IBD pathogenesis (Klasic et al. 2018).

8.6 Conclusion

Even if glycosylation and epigenetics, taken separately, have received much attention, it is only in the last two decades that they have been connected. In particular, it is necessary to discover more about the physiology of the tissue- and cell-specific epigenetic regulation of glycosylation and the dysregulations that can occur, especially in the perspective of finding new therapeutic personalized drugs.

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Part II

Glycosylation in Disease

Glycosaminoglycans in Neurodegenerative Diseases

9

Weihua Jin, Fuming Zhang, and Robert J. Linhardt

Abstract

Glycosaminoglycans (GAGs) are linear polysaccharides that consist of alternating disaccharides sequences of uronic acids and/or galactose hexamino sugars most of which are sulfated. GAGs are ubiquitously expressed on the cell surface, in the intracellular milieu and in the extracellular matrix of all animal cells. Thus, GAGs exhibit many essential roles in a variety of physiological and pathological processes. The targets of GAGs are GAG-binding proteins and related proteins that are of significant interest to both the academic community and in the pharmaceutical industry. In this review, the structures of GAGs, their binding proteins, and analogs are presented that further the development of GAGs and their analogs for the treatment of neurodegenerative diseases agents.

Keywords

Glycosaminoglycans · Neurodegenerative diseases · Heparan sulfate · Alzheimer's disease · Parkinson's disease

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9.1 Introduction: Neurodegenerative Diseases and Glycosaminoglycans

Neurodegenerative diseases (NDDs) are a heterogeneous group of disorders that are characterized by the progressive degeneration of the structure and function of the central nervous system or the peripheral nervous system (Nature Springer 2020a). More people are living longer so that more people are of higher risk of being affected by NDDs. Research shows that genes and envi-

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ronment contribute to NDDs (Nature Springer 2020b), however, it is still generally unknown which gene or which compounds impact NDDs. There are limited medicines for the treatment of the physical or mental symptoms related with NDDs and there is an urgent need to uncover and improve our understanding of the causes and cures of NDDs.

NDDs include Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), prion diseases, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), ataxia telangiectasia, multiple sclerosis, spinocerebellar ataxia (SCA), HIV-associated neurocognitive disorders (HAND), Pick's disease, Krabbe's disease, Kennedy's disease, primary lateral sclerosis (PLS), Cockayne syndrome, spinal muscular atrophy (SMA), tabes dorsalis, progressive supranuclear palsy (PSP), and Pelizaeus-Merzbacher disease (Appel et al. 1996; Hardy 2000).

There are millions of people suffering from NDDs throughout the world. Alzheimer's disease and Parkinson's disease are the most common NDDs. The number of Americans age 65 and older with Alzheimer's dementia is expected to grow from 5.8 million to 13.8 million by mid-century (Alzheimer's Association 2020). The number of deaths associated with AD was 0.12 million in 2018 making AD as the sixth leading cause of death in the United States and the fifth leading cause of death among 65 and older Americans (Alzheimer's Association 2020). This situation has been getting worse as the number of deaths resulting from AD has increased by 146.2% between 2000 and 2018 (Alzheimer's Association 2020). The total payments in 2020 for health care, long-term care, and hospice services for people aged 65 and older with dementia in the United State is estimated as $305 billion (Alzheimer's Association 2020). The number of people living with PD in the United States is predicted to rise from 0.93 million in 2020 to 1.2 million by 2030 (Marras et al., 2018). There are more than 10 million people, who are living with PD around the world. Like AD, the incidence of PD increases with age. An estimated 4% PD people are diagnosed before 50, which is 15 years younger than AD patients are identified (Marras et al. 2018). The combined direct and indirect cost of PD is approximately $52 billion per year in the United States (Marras et al. 2018).

The extracellular matrix (ECM) plays key roles in regulating the development, function, and homeostasis of all eukaryotic cells (Mouw et al. 2014; Nita et al. 2014; Barros et al. 2011; Vieira et al. 2018; Song and Dityatev 2018). The diverse functions of tissues are reflected and facilitated through the complex chemical composition and organization of ECM, which result from a biochemical and biophysical interplay between the various cells in each tissue and the evolving microenvironment (Mouw et al. 2014; Naba et al. 2012). In the central nervous system, ECM contains basal lamina, proteoglycans (PGs), collagens, fibronectin, and elastin (Mouw et al. 2014; Cui et al. 2013; Lau et al. 2013; Hubert et al. 2009; Barros et al. 2011; Ma et al. 2020). These components are also regulated by biochemical mediators, such as interleukins, arachidonic acid and derivatives, interferons, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF), and epidermal growth factor (Carmen et al. 2019). Therefore, the dysfunction of ECM in the brain leads to neurodevelopment disorders, psychiatric dysregulation, and neurodegenerative diseases (Wang and Ding 2014; Ariga et al. 2010; Hillen et al. 2018; Fawcett et al. 2019).

The most important components of the ECM are proteoglycans (PGs). PGs are a subset of heavily glycosylated glycoproteins. The "core proteins" include decorin, versican, testican, perlecan, bikunin, neurocan, aggrecan, brevican, fribromodulin, and lumican with their covalently attached carbohydrate portion being glycosaminoglycans (GAGs) (Timpl 1989; Carmen et al. 2019; Ariga et al. 2010; Wang and Ding 2014; Castillo et al. 1997; Snow et al. 1995; Smith et al. 2015; Hayes and Melrose 2018; Volpi 2006).

GAGs are a family of unbranched polysaccharides, including a repeating disaccharide unit (Fig. 9.1). The disaccharide unit usually contains an amino sugar (*N*-acetylglucosamine [GlcNAc] or *N*-acetylgalactosamine) (GalNAc) along with an uronic sugar (glucuronic acid [GlcA] or iduronic acid [IdoA]) or galactose (Gal)

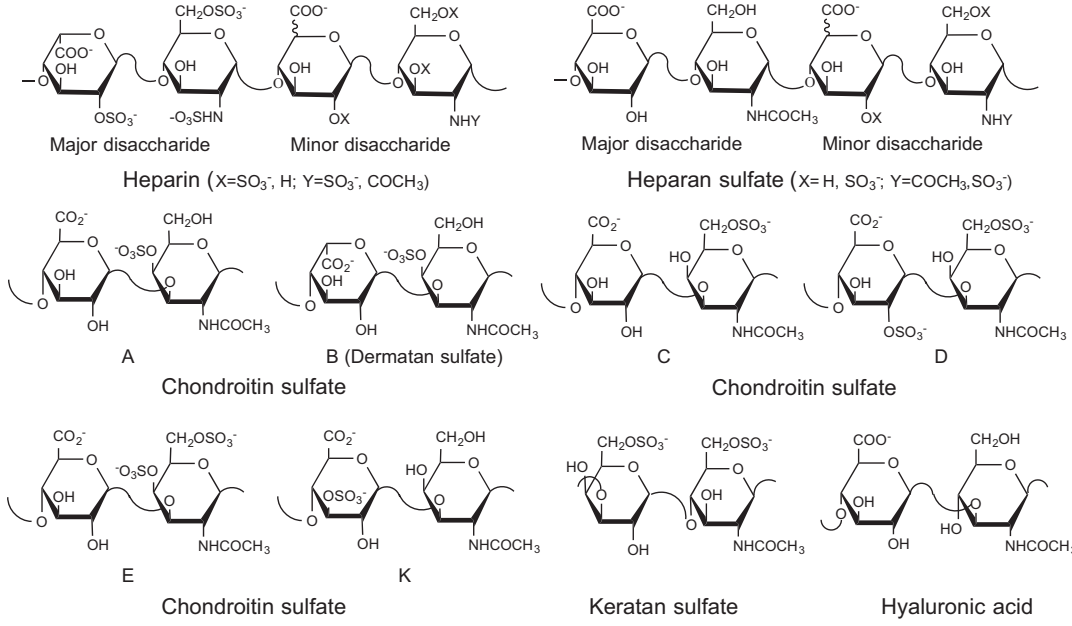

Fig. 9.1 Chemical structures of various glycosaminoglycans

(Constantopoulos and Dekaban 1975). The differences among GAGs were attributed to the type of monosaccharides, glycosidic linkage, and sulfation position and level. In this chapter, we present the importance of GAGs in the occurrence, development, and treatment of NDDs, particularly in AD.

9.2 Heparan Sulfate in Neurodegenerative Diseases

Cell surface heparan sulfate proteoglycans (HSPGs) act as coreceptors for an extensive and structurally diverse range of extrinsic effector proteins and many of these regulatory signals in the microenvironment of cells converge on HSPGs (Gallagher 2015; Celesia 1991; Cui et al. 2013; Fukuchi et al. 1998; Leonova and Galzitskaia 2015; Mahley 1996; O'Callaghan et al. 2018; van Horssen et al. 2003; Zhang et al. 2014). Heparan sulfate (HS) is the carbohydrate or GAG component of HSPGs. HS is synthesized in the Golgi. HS is comprised of an uronic acid (GlcA or IdoA) and a glucosamine (GlcN) residue (Fig. 9.1). Sulfate substitution is found on the amine group and at different hydroxyl groups, providing a variety of different disaccharide units in HS. The structural variation in HS is attributed to a variety of sulfotransferases and C5 epimerase (Li and Kusche-Gullberg 2016; Xu et al. 2011; Peterson et al. 2009; Presto et al. 2008; Kero et al. 2018; Li et al. 2003; Bullock et al. 1998). Glycosyl transferases (or polysaccharide syntheses) control the length of an HS chain and sulfotransferases control the sulfate patterns of saccharide residues and C5 epimerase converts GlcA into IdoA.

Each particular cell type or tissue shows the specific expression of HS metabolic machinery, leading to different sulfate signatures that can vary as a consequence of aging, tissue injury, or disease (Maiza et al. 2018). These changes in composition and sequence impact the interaction between HS and numerous proteins or peptides that are known as heparan sulfate binding proteins (HSBP) (Li and Kusche-Gullberg 2016; Xu and Esko 2014). In addition, HS can protect HSBP from proteolytic degradation, increasing the bioavailability of amyloidogenic proteins, and also in prompting their aggregation (Maiza et al. 2018; Ancsin 2003; Zhang and Li 2010; Kisilevsky et al. 2007; van Horssen et al. 2003).

Protein aggregation is a process in which misfolded proteins interact together to form well-structured fibrils, leading to the formation of filaments, known as amyloids (Maiza et al. 2018). Accumulation of amyloids in the brain tissue is related to many neurodegenerative diseases, including AD, PD, and HD (Som Chaudhury and Das Mukhopadhyay 2018; Ross and Poirier 2004; Collinge 2016; Peng et al. 2020).

AD is characterized by two major types of brain lesions and involves two main proteins, Aβ and Tau. Aβ forms amyloid plaques by extracellular accumulation while Tau makes neurofibrillary tangles through intraneuronal accumulation (Scheltens et al. 2016).

Snow et al. (1990) first described HS implication in amyloid plaque formation in the brains of AD patients. Many studies have subsequently demonstrated that HS plays a vital role in the aggregation of Aβ peptides (Castillo et al. 1997; Snow et al. 1994; Bame et al. 1997; Cotman et al. 2000; van Horssen et al. 2003; Patey 2006; O'Callaghan et al. 2008; Geneste et al. 2014; Fu et al. 2016; Liu et al. 2016; Nguyen and Rabenstein 2016; Vera et al. 2017; Maiza et al. 2018; Wesen et al. 2018). For example, the overexpression of HS degrading heparanase, decrease the number of Aβ amyloid plaques without altering the production and proportion of Aβ40 and Aβ42 peptides, which are derived from the sequential cleavage of amyloid precursor protein by β- and γ-secretases (Jendresen et al. 2015; Nagai et al. 2007; Schworer et al. 2013; Cheng et al. 2014). Additionally, HS interacts with residues 12–18 (VHHQKLV) in Aβ40 and Aβ42 and also breaks the anionic bridge of Aβ42 between lysine 28 and alanine 42, leading to the acceleration of the aggregation process (Zhang et al. 2014). Moreover, sulfation content and pattern of HS also influence Aβ peptide aggregation. Highly sulfated HS deposits in both Aβ40 and Aβ42 amyloids while under sulfated HS only deposits in Aβ40 amyloid, suggesting that high sulfation content will prompt the aggregation of the Aβ42 peptide in the AD's brains (Maiza et al. 2018). On the aspects of sulfation pattern, it was found that different isoforms of amyloids require an HS with different sulfation patterns to promote the interaction between HS and Aβ. For example, Aβ amyloid fibrils require *N*- and 2-*O*-sulfation while Aβ monomer requires 6-*O*-sulfation (Lindahl et al. 1999). The secondary structure of Aβ peptides is impacted by the degree of polymerization (DP), disaccharide sequences, sulfate content, and sulfation pattern suggesting that HS is involved in the Aβ aggregation process in the AD brain (Maiza et al. 2018).

Unlike Aβ, Tau is a soluble protein, which does not aggregate *in vitro* without the incorporation of polyanionic molecules. An abnormally phosphorylated Tau protein (P-Tau) is found in AD brain. P-Tau deposits inside neurons along with HS forming paired helical filaments and grow into neurofibrillary tangles (NFTs). This suggests that HS is part of the Tau aggregation process and also regulates the P-Tau aggregation process (Snow et al. 1990; Konno et al. 2004; Iqbal et al. 2010). In addition, HSPGs also participate in the spreading of Tau-related proteins (Holmes et al. 2013; Goedert and Spillantini 2017). HS plays a vital role in the process of Tau misfolding, phosphorylation, aggregation, and spreading in the AD brain. This has been a hot research topic in elucidating the role of HS structure on Tau pathology. For example, the 3-*O*-sulfated HS might act as a molecular chaperone for abnormal Tau phosphorylation (Sepulveda-Diaz et al. 2015; Alavi Naini and Soussi-Yanicostas 2018; Thacker et al. 2014; Zhao et al. 2019). In addition, 6-*O*-sulfated heparan sulfate is required for interaction with Tau and also for Tau internalization (Zhao et al. 2017; Rauch et al. 2018). Moreover, it was reported that knockouts of HS extension enzymes (polysaccharide synthases) exostosin 1 (*EXT1*), exostosin 2 (*EXT2*), exostosin 3 (*EXT3*), *N*-deacetylase, *N*-sulfotransferase (*NDST1*), and 6-*O*-sulfotransferase (*HS6ST2*) significantly reduce Tau uptake, suggesting that Tau aggregates display specific interactions with HSPGs that depend on chain length and the position of sulfate groups (Stopschinski et al. 2018; Garcia et al. 2017). HS accumulates with protein deposits not only in AD, but also in other neurodegenerative diseases, which is summarized in Table 9.1.

Table 9.1 Neurodegenerative disease-related GAG-binding proteins

Types	Related proteins	Neurodegenerative diseases	References
GAGs	Aβ	Alzheimer's disease	Dawkins and Small (2014), Castillo et al. (1999), Madine et al. (2009), Genedani et al. (2010), Huynh et al. (2012), Stewart et al. (2016, 2017), Stopschinski et al. (2018), Takase et al. (2016), Timmer et al. (2010), van Horssen et al. (2003)
GAGs	Amyloid protein precursor (APP)	Alzheimer's disease	Small et al. (1994), Hamazaki (1987), Heegaard (1998), Coria et al. (1988), Kalaria et al. (1991), Danielsen et al. (1997), Lazarov and Demars (2012), Nalivaeva and Turner (2013), Castillo et al. (1999)
GAGs	Androgen receptor protein	Kennedy's disease	Rusmini et al. (2016), Hashimura et al. (2005)
–	Ataxin-1	Spinocerebellar Ataxia	Sullivan et al. (2019), Trott and Houenou (2012)
GAGs/ analogues	β, γ-secretase	Neurodegenerative diseases	Sun et al. (2017), Yang et al. (2019), Zhou et al. (2019), Leveugle et al. (1997), Patey et al. (2006, 2008)
GAGs/ analogues	Enzyme: Heparanase, glucuronate transferase	Neurodegenerative diseases	Ariga et al. (2010), Li et al. (2005), Lauri et al. (1999), Kisilevsky et al. (2007)
HS/HP/ CS/DS	Extracellular matrix proteins	Neurodegenerative diseases	Gallagher (2015), Alberts et al. (2015), Gundersen (1987), Rivas et al. (1992), Cescon et al. (2016), Streit et al. (1993), Snow et al. (1996)
HS/HP	Fibroblast growth factor, fibroblast growth factor-2	Neurodegenerative diseases	Woodbury and Ikezu (2014), Zhang et al. (2019), Bellucci et al. (2007)
HS/HP/ CS/DS	Glial cell line-derived neurotrophic factor (GDNF)	Parkinson's disease	Grondin et al. (2019), Gallagher (2015), Djerbal et al. (2017), Sugahara and Kitagawa (2000)
GAGs	Growth factors	Alzheimer's disease	Huynh et al. (2019)
–	Huntingtin protein	Huntington's disease	Koyuncu et al. (2017)
GAGs/ analogues	Lipoproteins: ApoE, ApoB, lipoprotein lipase	Alzheimer's disease	Dong et al. (2001), Arai et al. (1999), Lam et al. (2011), Mahley and Ji (1999), Gonzales et al. (2013), Gordts et al. (2014), Stanford et al. (2009, 2010), Li et al. (2005), Eisenberg et al. (1992), Strittmatter et al. (1993), Corder et al. (1994), Shuvaev and Siest (2000)
CS/DS	Midkine/ pleiotrophin	Neurodegenerative diseases	Deepa et al. (2002), Nandini et al. (2004), Matsumoto et al. (1994), Kadomatsu (2005), Kadomatsu and Muramatsu (2004), Ueoka et al. (2000), Mizumoto et al. (2013), Yasuhara et al. (1993), Solera et al. (2016)
HP	Nardilysin	Alzheimer's disease	Bernstein et al. (2013)
HS/HP	PrP^{Sc}	Prion disease	Ben-Zaken et al. (2003), Warner et al. (2002), Horonchik et al. (2005)
HS/HP	Selenoprotein P	Alzheimer's disease	Solovyev et al. (2018)
KS	Sulfotransferase "GlcNAc6ST1"	Alzheimer's disease	Zhang et al. (2017)
HP	Superoxide dismutase	Amyotrophic lateral sclerosis	Sangwan and Eisenberg (2016), Mizuguchi et al. (2010), Zhao et al. (2014)

(continued)

Table 9.1 (continued)

Types	Related proteins	Neurodegenerative diseases	References
HS/HP	Syndecan-1 and 3	Neurodegenerative diseases	van Horssen et al. (2003), Stanford et al. (2009), Kaksonen et al. (2002)
GAGs/ analogues	α-Synuclein protein	Alzheimer's disease, Parkinson's disease	Cohlberg et al. (2002), Lehri-Boufala et al. (2015), Mehra et al. (2018), Stopschinski et al. (2018), van Horssen et al. (2004), Holmes et al. (2013)
GAGs	Tau protein	Alzheimer's disease, Pick's disease, progressive supranuclear palsy	Alavi Naini and Soussi-Yanicostas (2018), Goedert et al. (1996), Snow et al. (1990), Huynh et al. (2019), Holmes et al. (2013)

9.3 Heparin in Neurodegenerative Diseases

Heparin (HP) (Fig. 9.1) has a high negative charge density (about 3.3 negative charges per disaccharide), especially compared to the related HS (about 0.8 sulfate groups per disaccharide in typical HS) (Weiss et al., 2017). HP is composed of 90% L-IdoA and 10% D-GlcA while HS consists of primarily GlcA (Capila and Linhardt 2002). Moreover, HP has an average molecular weight of about 15 kDa, ranging from 5 to 40 kDa while HS has a 30 kDa average molecular weight, ranging from 5 to 50 kDa. HP has a lower molecular weight, a higher sulfate content, and a higher IdoA content than HS. HS is ubiquitously expressed in all animal cells while HP is produced and stored selectively in the secretory granules of connective-tissue mast cells (Weiss et al. 2017; Guyton and Hall 2006).

HP, an anticoagulant drug, is used as a surrogate for HS in most research (Xu and Esko 2014) because HP is commercially available in ton quantities while HS is only available in gram quantities. Moreover, the higher sulfation level in HP results in its tight binding to HSBPs. Currently, HP has been reported to bind to more than 300 secreted and membrane associated human proteins and, in most cases, the natural ligand is HS (Xu and Esko 2014; Ori et al. 2011). These HSBPs can be divided into several major categories, chemokines and cytokines (~60), growth factors and morphogens in development and tissue repair (~50), blood coagulation factors (~25), extracellular structural proteins (~25), complement proteins (~20), single-transmembrane signaling receptor (~15), cell adhesion proteins (~10), and other proteins (5–10), such as proteins in intracellular granules, lipid-binding proteins, and "helper proteins" (Xu and Esko 2014). These HSBPs play key roles in NDDs (summarized in Table 9.1). There are many excellent reviews (Alavi Naini and Soussi-Yanicostas 2018; Muramatsu 1993; Stutzmann et al. 2002; Ma et al. 2007; Dudas et al. 2008; Bergamaschini et al. 2009; Ariga et al. 2010; Dudas and Semeniken 2012; Szczubialka et al. 2012; Wang and Ding 2014; Woodbury and Ikezu 2014; Lima et al. 2017) on HP or HS and NDDs.

9.4 Chondroitin Sulfate and Dermatan Sulfate in Neurodegenerative Diseases

Chondroitin sulfate (CS) was first found from cartilage by Fisher and Boedecker in 1861, isolated in purer form by Krukenburg in 1884, elucidated the full structure of CS by Levene and Forge until 1915 (Djerbal et al. 2017). The different forms are attributed to sulfation pattern and epimerization pattern, which divided CS into six forms (Fig. 9.1), namely chondroitin-4-sulfate (CS-A), chondroitin-6-sulfate (CS-C), chondroitin-2,6-sulfate (CS-D), chondroitin-4,6-sulfate (CS-E), chondroitin-3-sulfate (CS-K), and chondroitin sulfate B (CS-B), which is the product of uronic acid epimerization and also named dermatan sulfate (DS).

CS is found in the ECM, at the cell surface, associated with the plasma membrane in most

animal tissues, and also, in the case of CS-E, in the intracellular granules of certain cells like mast cells (Yamada et al. 2011; Stevens et al. 1988; Thompson et al. 1988; Farrugia et al. 2016). The expression of CS is different in different tissues and the highest level is found in the ECM in cartilage and in the central nervous system (Djerbal et al. 2017). CS is synthesized in the Golgi. Like HS, CS is also linked to the "core proteins" to form chondroitin sulfate proteoglycans (CSPGs) in the ECM and at the cell surface, and are involved in the formation, development, and maintenance of brain morphology and function (Djerbal et al. 2017; Egea et al. 2010; Kastana et al., 2019; Khan et al. 2020; Kwok et al., 2008; Maeda et al. 2010; Malavaki et al. 2008; Malmstrom et al. 2012; Mikami and Kitagawa 2013, 2015; Purushothaman et al. 2012; Rani et al. 2018; Rauvala et al. 2017; Avram et al. 2014; Carulli et al. 2005; Maeda 2010; Oohira et al. 2004; Sugahara and Mikami 2007). The ratio of CS to HS in the central nervous system (CNS) is 9:1 while it is 7:3 in the perineuronal net (PNN) matrix. There are many proteins that interact with various forms of CS (depending on the different sulfate content, pattern, and uronic acid epimerization) to accomplish their functions in promoting growth, differentiation, guidance, and plasticity. These proteins can be divided into several categories (Djerbal et al. 2017), growth factors (neurotrophic factors, FGF, and Midkine and pleiotrophin), receptors (receptor protein tyrosine phosphatases and Nogo receptors NgR1 and NgR3), cell adhesion molecules, guidance proteins (semaphoring), extracellular matrix proteins (collagen VI, laminin and fibronectin), and pathological protein (amyloid precursor protein). These CS binding proteins and their functions in the NDDs are summarized in Table 9.1.

9.5 Hyaluronic Acid in Neurodegenerative Diseases

Hyaluronic acid (HA) was firstly discovered by Meyer and John Palmer in 1934 from vitreous body in cow's eye. As the simplest structure among GAGs, HA is composed of GlcA and GlcNAc (Fig. 9.1) with a molecular weight range from 5000 to 20 million Da (Carmen et al. 2019; Toole 2004). HA is synthesized on the cell membrane without any modification by a class of integral membrane proteins, named hyaluronan synthases (HAS1, HAS2, and HAS3) (Toole 2004). More than half of HA is localized in the skin, about 25% is in the skeleton and supporting structures and less than 10% is in the skeletal muscle (Reed et al. 1988).

The sulfate moiety is necessary for the formation of amyloid fibrils, as confirmed the lack of fibril formation in the presence of HA (Ariga et al. 2010) and it is suggested that HA might inhibit the fibril formation and be useful for treating AD. There is a positive correlation between HA accumulation and AD neuropathology, suggesting that HA synthesis and metabolism play a role in AD (Reed et al. 2019). More specifically, HA reduces Aβ42 oligomer uptake and supports neuron cell survival (Bejoy et al. 2018). During the progression of AD, the abolishing of axonal-localization of HAS1 and an increased expression of HAS3 results in the upregulation of short-chain HA production and the reorganization of the ECM, providing biochemical and physical support to aggrecan-based perineuronal nets and regulation of neuronal plasticity (Li et al. 2017).

9.6 Keratan Sulfate in Neurodegenerative Diseases

Keratan sulfate (KS) was first isolated from the cornea and is found in brain, skeletal, and nervous tissues (Meyer et al. 1953; Funderburgh 2000; Kleene and Schachner 2004; Lindahl et al. 1996). KS consists of disaccharide unit [Gal and GlcNAc], sulfated at C6 of both Gal and GlcNAc residues (Fig. 9.1). KS is covalently attached to a "core protein" through its reducing end in both an *O*-linkage to *N*-acetylated galactosamine (GalNAc) or *N*-linkage Fuc-Man-GlcNAc-linked oligosaccharides and can be terminated with a sialic acid residue or a Gal or GalNAc at the non-

reducing end of KS (Funderburgh 2000). KS is synthesized by Golgi-resident enzymes, such as galactosyltransferase, *N*-acetylglucosaminyl transferase, and Gal/GlcNAc/GalNAc sulfotransferases (Uchimura and Rosen 2006; Christner et al. 1979; Funderburgh 2000). KS plays an indispensable, suppressive role in ALS pathology progression by microglial activation and proliferation, suggesting that KS might be a new target for the treatment of ALS (Foyez et al. 2015; Hirano et al. 2013). Deficiency of a sulfotransferase "GlcNAc6ST1" synthesizing sialylated KS can modulate AD pathology, suggesting that GlcNAc6ST1 also might be a good target (Zhang et al. 2017).

9.7 Glycosaminoglycans and Their Analogues in Neurodegenerative Diseases

Aβ is an important target for treating AD. Although Aβ self-aggregates to form amyloid fibrils in vitro, amyloid aggregation and fibril formation can be enhanced in the presence of PGs or GAGs (Castillo et al. 1998, 1999). Their binding sites in Aβ at the 13–16 amino acid region (His-His-Gln-Lys), particular of His 13, are important for the interaction between GAGs with Aβ (Ariga et al., 2010). The sequence of the effectiveness of GAGs on prompting fibril formation is HP > HS > CS = DS. In HP the sequence of the effectiveness based on sulfate content and position is HP > *N*-desulfated *N*-acetylated HP > completely desulfated *N*-desulfated HP > completely desulfated *N*-acetylated HP (Castillo et al. 1998, 1999). Fibril formation can be inhibited by low molecular weight heparin (LMWH, MW 4000–6000), suggesting that GAG analogs might be used in inhibiting fibril formation. Research on NDDs have examined "neuroparin," a low molecular weight GAG (Ma et al. 2003; Dudas et al. 2008), a specific disaccharide (CSPG-DS) (Rolls et al. 2004), and HP oligosaccharides (Ariga et al. 2010). There are also some Aβ related targets, including ApoE and β-secretase (Shuvaev and Siest 2000; Leveugle et al. 1997; Patey et al. 2006, 2008). There are also some GAG analogs, such pentosan polysulfate, that have been used to target Aβ (Ariga et al. 2010).

Tau is also an important target for treating AD. Unlike Aβ, Tau needs polyanionic molecules to aggregate. In addition, Tau phosphorylation plays an important role in AD progression. Therefore, Tau is an important target for curing AD. In terms of GAGs and their analogs that influence Tau phosphorylation by the proline-directed protein kinases NCLK, GSK3β and MAP kinase, HP, dextran sulfate, pentosan polysulfate, and HS show efficacy, while KS, HA, and dextran and poly-L-glutamic acid are ineffective. It is interesting to note that CS and DS had intermediate effects when Tau was phosphorylated by NCLK and GSK3β, but no effect when Tau was modified by MAP kinase (Hasegawa et al. 1997). There are a number of reviews on the GAGs analogs and their biological activities (Mende et al. 2016; Morla 2019; Zhang et al. 2020; Fraser et al. 2001; Arlov and Skjak-Braek 2017; Coombe and Kett 2012).

9.8 Conclusion

This chapter described the importance of GAGs in NDDs. The data presented probably underrepresents the significance of GAGs, as GAGs interact with many proteins. In addition, there are many GAGs attached to a variety of core proteins. These core proteins can also exhibit important roles in the progression of NDDs. There is still much to uncover about the role of GAGs in NDDs. The development of effective compounds to target the interactions of GAGs for the treatment of NDDs still represents a formidable challenge.

Compliance with Ethical Standards

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Glycosylation in Autoimmune Diseases

10

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Abstract

Autoimmune diseases are accompanied by changes in protein glycosylation, in both the immune system and target tissues. The best-studied alteration in autoimmunity is agalactosylation of immunoglobulin G (IgG), characterized primarily in rheumatoid arthritis (RA), and then detected also in systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and multiple sclerosis (MS). The rebuilding of IgG *N*-glycans in RA correlates with the relapses and remissions of the disease, is associated with physiological states such as pregnancy but also depends on applied anti-inflammatory therapy. In turn, a decreased core fucosylation of the whole pool of IgG *N*-glycans is a serum glycomarker in autoimmune thyroid diseases (AITD) encompassing Hashimoto's thyroiditis (HT) and Grave's disease (GD). However, fucosylation of anti-thyroglobulin IgG (an immunological marker of HT) was elevated in HT serum. Core fucosylation of IgG oligosaccharides was also lowered in MS and SLE. In AITD and IBD, chronic inflammation T lymphocytes showed the reduced expression of *MGAT5* gene encoding β1,6-*N*-acetylglucosaminyltransferase V (GnT-V) responsible for β1,6-branching of *N*-glycans, which is important for T cell receptor activation. Structural changes of glycans have a profound effect on the pro-inflammatory activity of immune cells and serum immune proteins, including IgG in autoimmunity.

Keywords

Glycosylation · Core fucosylation · Galactosylation · Sialylation · β1,6-branching · Autoimmune diseases

10.1 Introduction

Distinguishing of self from foreign antigens is a crucial aim of both innate and adaptive (acquired) immune responses (Chaplin 2010). A state in which the dysregulated adaptive response is turned against self-antigens refers to autoimmunity, and disorders caused by autoimmune response are defined as autoimmune diseases (Katsorida and Moutsopoulos 2001). Modifications of self-antigens, mainly protein

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epitopes, but also oligosaccharides, lipids, and nucleic acids, can lead to breakage of immune tolerance, which results in the production of autoantibodies belonging mainly to immunoglobulins G class (IgG) (Elkon and Casali 2008). Antigenic properties of proteins are well described and among autoantigens, protein epitopes are the most often identified as the targets of an autoimmune response. In immunity of carbohydrates, the immune response is elicited mainly against monosaccharides and oligosaccharides of non-human origin (Kappler and Hennet 2020). Blood group determinants, mainly ABO(H) system, are the well-known examples of oligosaccharide antigenicity (Delves 1998) with antibodies to ABO antigens generated after contact of immune cells with intestinal microbiota (Kappler and Hennet 2020). Glyco-epitopes (glycotopes), the sugar epitopes on glycoconjugates, also contribute to human autoimmunity. Carbohydrate-specific epitopes have been identified in autoimmune diseases such as multiple sclerosis (MS) (GM1, GM2, and G7 gangliosides, galactocerebrosides) and inflammatory bowel disease (IBD) (serum fucosylated oligosaccharides) (Kappler and Hennet 2020). A hypothesis coined by Sandra McLachlan and co-workers assumes that more abundant *N*-glycosylation of a subunit A of the thyrotropin receptor (TSHR) expressed on human thyrocytes in comparison to other placental mammals is the major factor contributing to THSR immunogenicity and development of Graves' disease (GD) in human (McLachlan et al. 2011).

Apart from antigenic properties of oligosaccharides and their impact on the development of autoimmune diseases, a growing number of research results has provided the well-established evidence that autoimmunity is accompanied by the changes in glycosylation of cellular and secreted proteins (Alavi and Axford 2008; Lauc et al. 2016; Maverakis et al. 2015) produced by both immune system (Chien et al. 2018; Kozłowska et al. 2018) and target tissues (Ząbczyńska et al. 2018). The changes of glycosylation and an impact of glycan modifications on autoimmunity have been studied on human serum or tissue samples, and on several animal models of T-cell-mediated autoimmune diseases, respectively (Chien et al. 2018). This chapter provides an insight into the dysregulation of glycosylation in the most common autoimmune diseases based on the latest data obtained for human samples, animal, and *in vitro* models.

10.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases which primarily affects the synovial joints. Chronic, progressive inflammation can lead to joint destruction, deformation, and eventual disability. RA patients in some cases present also distinct manifestations affecting the heart, lungs, skin, eyes, kidneys, and blood vessels. The etiology of the disease is considered multifactorial with the combination of genetic and environmental risk factors. There are several types of autoantibodies associated with RA and some of them have important diagnostic and predictive value. These include anti-citrullinated protein/peptide antibodies (ACPA), rheumatoid factor (RF), anti-keratin antibodies (AKA), anti-perinuclear factor (APF), anti-fibronectin antibody (AFA), anti-mutated citrullinated vimentin (anti-MCV) antibody, anti-Sa antibody, anti-glucose-6 phosphate isomerase (anti-GPI) antibody, anti-carbamylated protein (anti-CarP) antibody, anti-acetylated protein antibody, anti-nuclear antibody (ANA), anti-heterogeneous nuclear ribonucleoprotein (anti-hnRNP/RA33) antibody, anti-Bip antibody and anti-calcitostatin antibody (ACAST), and anti-CII antibody (Fang et al. 2019). ACPA and RF are the most studied autoantibodies in terms of their prognostic value as well as involvement in the disease pathogenesis. It is worth noting, however, that RF is not specific for RA as it is also common in chronic and infectious diseases, whereas ACPA is strongly associated with arthritis (Kempers et al. 2018).

Rheumatoid arthritis is one of the most studied autoimmunity in terms of the role of glycosylation as a causative factor as well as its biomarker and prognostic utility. The most studied issue in this field so far is the comparative analyses of serum IgG glycosylation patterns of RA patients and healthy controls. The early studies of Raj Parekh and co-workers (Parekh et al. 1985)

showed that although serum-derived total IgGs of RA patients do not possess novel *N*-linked oligosaccharides comparing to healthy individuals, they are significantly agalactosylated. These changes were postulated to create a shift in the population of IgG towards those possessing diantennary *N*-glycans with terminal *N*-acetylglucosamine (GlcNAc) residue on one or both arms. This observation was further demonstrated by numerous data basing on the analyses of several human cohorts and populations (Bond et al. 1997; Gindzienska-Sieskiewicz et al. 2007; Martin et al. 2001; Watson et al. 1999). The phenomenon of IgG agalactosylation during the course of RA has been demonstrated to have a prognostic value. The correlation between the disease progression state and the downregulation of terminal galactose (Gal) on IgGs was found over 20 years ago (Lacki et al. 1996). Moreover, more recent studies have shown that low galactosylation of serum IgG associates with a higher risk of RA diagnosis in the future (Gudelj et al. 2018). The latter observation, showing that RA-associated agalactosylation of IgGs proceeds the disease outcome, suggests the causative importance of this glycosylation modification. Although the link between the downregulation of terminal Gal content on IgGs and the disease development is still under investigation, some observations seem to confirm the potential existence of this relationship. First of all, it was proved that changes in the *N*-glycans galactosylation correlate with the relapses and remissions of the disease, associated with physiological states such as pregnancy (Bondt et al. 2013; Van de Geijn et al. 2009), but also as a result of anti-inflammatory therapy with methotrexate and/or infliximab (anti-tumor necrosis factor-α monoclonal antibody, TNFα mAb) (Croce et al. 2007; Gińdzieńska-Sieśkiewicz et al. 2016; Pasek et al. 2006). More direct evidence was presented by Thomas Rademacher and coworkers (1994) showing that in murine collagen-induced arthritis, the nonpathogenic IgG autoantibodies fraction could be converted into pathogenic one by the enrichment with the agalactosyl IgG glycoforms.

The agalactosylation of complex glycans on IgGs is not the only modification described in RA patients to date, but it has been reported most often. Downregulation of sialylation is another change present in the literature (Parekh et al. 1985; Van de Geijn et al. 2009), but surprisingly it was also reported that during pregnancy galactosylation is associated with improvement of RA independently of sialylation (Bondt et al. 2013). Additionally, in early studies of Ivan Gornik and co-workers, based on plant lectin staining it was shown, that the fucosylation of IgG heavy chain is significantly increased in RA patients (Gornik et al. 1999). It was found also that IgGs from RA sera express sialyl Lewis x (sLex) antigens in correlation with disease activity (Goodarzi et al. 1998). Finally, thanks to the development and broader accessibility of modern analytical techniques for glycan analysis such as hydrophilic interaction ultra performance liquid chromatography (HILIC-UPLC) and mass-spectrometry, the IgG glycosylation changes accompanying RA has been studied in more details, taking into consideration an individual glycan structure and/or glycan epitopes (Huang et al. 2017; Su et al. 2020; Sun et al. 2019). All these studies, in general, have confirmed the previous observations of the downregulation of galactosylation and sialylation and upregulation of fucosylation of IgGs from RA patients' sera, suggesting some of these changes as potential biomarkers of the disease.

The data presented above showing clear evidence of changed IgG glycosylation in the course of RA are particularly interesting because of the role of *N*-glycosylation of the Fc region in the modulation of antibody functionality. This phenomenon is also broadly discussed in this book (see Chap. 17). In this context, the interaction of the antibody Fc region with different subsets of Fcγ receptors, which in part is regulated by Asn297-linked glycan moiety, seems to be important. However, the direct evidence suggesting the involvement of RA-associated IgG glycoforms in autoimmunity is quite limited. It was shown, for example, that agalactosylation of RA-associated IgGs can support the interactions with complement through binding to the mannose-binding protein (MBP) and thus possibly promoting chronic inflammation within the joints (Malhotra et al. 1995).

Besides a wide range of studies concerning the total serum IgG glycosylation in RA patients,

there are also some quite interesting threads in research, regarding the role of glycosylation in a slightly different aspect. For example, the glycosylation of RA-associated autoantibodies has been studied. The analyses of ACPA-IgG Fc region is in line with general observations of pro-inflammatory glycosylation changes of total serum IgG, presented above (Rombouts et al. 2019). Adding to that, interesting data have been published on a predictive role of ACPA-IgG variable domain glycosylation prior to the onset of RA (Hafkenscheid et al. 2019). The functional role of these glycan modifications is now started to be discussed mainly in the context of their hypersialylation (Kempers et al. 2018; Wu et al. 2020). Some other studies describe the glycosylation of RA-derived immune complexes, showing an elevated level of GlcNAc (Bond et al. 1995). Rheumatoid arthritis synovial tissue was also studied in terms of glycans modifications, showing up-regulation of α1,2-linked fucosylation, and fucosyltransferase 1 (Fut1) in synovial fibroblast was postulated to have an important role in angiogenesis, leukocyte-synovial fibroblast adhesion, and synovial fibroblast proliferation (Isozaki et al. 2014). Finally, the other-than-IgG serum glyco-biomarkers are also an object of interest in current research. In this context, the elevated fucosylation of α_1-acid glycoprotein is postulated to have some importance, supplementary to traditional disease biomarkers described so far (Rydén et al. 2002).

10.3 Autoimmune Thyroid Diseases

Hashimoto's thyroiditis (HT) and GD belong to organ-specific autoimmune thyroid diseases (AITD), with one of the highest prevalence rates reaching 5% of the population (Antonelli et al. 2015; Fröhlich and Wahl 2017). The immunological markers of AITD are serum IgGs against three thyrocyte proteins which play key roles in thyroid endocrine functions. Thyroglobulin (Tg) is a high molecular weight protein secreted to the lumen of the thyroid follicles where serves to the synthesis of thyroid hormones, L-triiodothyronine (T3) and L-thyroxine (tetraiodothyronine, T4) (Di Jeso and Arvan 2016). One of the crucial enzymes involved in thyroid hormone biosynthesis is thyroid peroxidase, named also thyroperoxidase (TPO), responsible for the oxidation of iodide in the presence of hydrogen peroxide, iodination of tyrosine residues within Tg polypeptide chain to 3-monoiodothyrosine (MIT) and 3,5-diiodothyrosine (DIT), as well as coupling of these iodinated tyrosine residues to form T3 and T4 (Carvalho et al. 2000). The production of thyroid hormones is controlled by thyroid-stimulating hormone (thyrotropin, TSH) secreted by the anterior pituitary gland which affects thyroid follicular cells (thyrocytes) by a transmembrane receptor TSHR (Korta and Pocheć 2019). Auto-antibodies against Tg and TPO are characteristic for HT, and TSHR is the main antigen responsible for breaking immune tolerance in GD (Fröhlich and Wahl 2017). Thyroid autoantibodies in low concentrations are present also in healthy people (Kohno et al. 1991; Naito et al. 1990). Each of these thyroid-specific autoantigens and TSH are glycosylated, by attaching *N*-glycans to protein chains [reviewed in Ząbczyńska et al. 2018].

Glycosylation of the thyroid antigens and its role in thyroid physiology is quite well defined (Ząbczyńska et al. 2018) in contrast to an impact of glycosylation on AITD induction and progression of the disease which is poorly understood. As mentioned in the Introduction, *N*-glycosylation of the TSHR was suggested to contribute to the immunogenicity of this receptor and GD development, because the poorer glycosylation of the TSHR A subunit in other mammals than human is related to its lower immunogenicity and an absence of GD in this group of animals (McLachlan et al. 2011). Cell surface mannose receptor (ManR) on antigen-presenting cells (APCs) with a lectin activity was suggested to participate in thyroid autoimmune responses to Tg and TSHR, due to ManR ability to bind heavily glycosylated TSHR A subunits and Tg released from thyrocytes and captured by APCs residing in the thyroid gland or lymph nodes (Chazenbalk et al. 2005).

Recent studies have provided data on the changes in serum protein glycosylation characteristic for AITD. The first research performed using lectin-enzyme-linked immunosorbent assay (lectin-ELISA) (Zhao et al. 2013), a panel

of 94 lectins in microarray technology and matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF-MS/MS) (Yuan et al. 2015) focused on glycosylation of anti-Tg IgG (TgAb) in AITD and papillary thyroid carcinoma (PTC). The results revealed significant differences in TgAb sialylation and fucosylation. Core fucosylation of TgAb is enhanced in HT autoimmunity in comparison to healthy donors (Zhao et al. 2013) and it is also upregulated in GD in comparison to HT (Yuan et al. 2015). The comprehensive profiling of glycosylation by lectins microarrays also showed the higher sialylation of TgAb in HT sera in relation to the control group (Yuan et al. 2015). The amount of sialic acid (Sia) evaluated by Elderberry lectin (EBL), was lower on TgAb in both AITDs than in PTC, the most frequent thyroid neoplasm (Zhao et al. 2013).

The analysis of serum glycome, separately for IgG, which is the most abundant serum glycoprotein, and the rest of glycosylated proteins, is a commonly used strategy enabling detection of signals from glycoproteins at lower concentrations and contributing to obtain more reliable and accurate results. A downregulation of *N*-glycan structures with core fucose in AITD sera in comparison to healthy individuals was the most significant alterations of IgG glycome identified in three different European cohorts by applying HILIC-UPLC and *Aleuria aurantia* lectin (AAL) blotting. Based on the data from genome-wide association studies (GWASs), *IKZF1* and *FUT8* genes were identified to be associated with the decrease of IgG core fucosylation in AITD. The expression of both *IKZF1* gene which encodes Ikaros family zinc finger protein 1, a transcription factor involved in chromatin remodeling and regulating lymphocyte differentiation, and *FUT8* gene encoding fucosyltransferase 8 responsible for α1,6-fucosylation of *N*-glycan core, is related to protein fucosylation (Martin et al. 2020). An increase of monosialylated tri-antennary *N*-glycans and disialylated diantennary *N*-glycans with antennary fucose was characteristic for glycoproteins in IgG-depleted HT sera as was shown by normal phase high-pressure liquid chromatography (NP-HPLC) analysis. A significant decrease of *Lens culinaris* agglutinin (LCA) staining of HT serum proteins indicating the reduction of α1,6-linked fucose and an increase of *Maackia amurensi*s II lectin (MAL-II) reaction in HT due to the elevated level of α2,3-sialylation in HT sera was demonstrated by lectin blotting (Ząbczyńska et al. 2020a). The various origin of serum proteins (most are secreted from liver, intestine and plasma cells) (Anderson and Anderson 2002) resulting in their tissue/organ-specific glycosylation may be the primary reason for the diverse alterations of glycan composition observed for IgG (Martin et al. 2020) and other serum proteins (Ząbczyńska et al. 2020a) in HT chronic inflammation.

Changes in *N*-glycan composition have profound effects on IgG activity by redirection its pro- and anti-inflammatory action (Shade and Anthony 2013). Thyroid tissue in AITD is destructed by antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), both mediated by anti-TPO (Le et al. 2015; Rebuffat et al. 2008). The functional analysis of differentially *N*-glycosylated IgG isolated from the sera of HT patients and healthy donors showed the diverse effect of antibody *N*-glycans in an *in vitro* model of ADCC and CDC. HT IgGs induced a higher cytotoxic effect on thyrocytes. The altered sialylation of IgG in HT affected thyrocyte lysis in both cytotoxicity models. Desialylation of IgG *N*-glycans, accompanied by exposure of Gal residues, enhanced ADCC intensity and decreased the CDC process (Ząbczyńska et al. 2020b).

Glycosylation in thyroid autoimmunity is also affected on immune cell proteins. The study on peripheral blood mononuclear cells (PBMCs), a heterogeneous cell population encompassing lymphocytes, monocytes, and dendritic cells, demonstrated a significant reduction of antennary α1,2-fucosylation of PBMC proteins in HT compared to healthy donors using *Ulex europaeus* agglutinin (UEA-I) in lectin blotting (Martin et al. 2020). The ongoing research on the CD4+ T cell population sorted from human blood has provided interesting results showing that the expression of genes encoded glycosyltransferases FUT8, β1,6-*N*-acetylglucosaminyltransferase V (GnT-V), α2,6-sialyltransferase (ST6Gal1), and α2,3-sialyltransferase (ST3Gal4) is subject to change in both AITDs (Korta et al. 2019;

Ząbczyńska et al. 2019). The transcript level of *FUT8*, *MGAT5* encoded GnT-V, and *ST6Gal1* was significantly reduced in CD4+ CD25+ T cells from HT (Korta et al. 2019) and GD (Ząbczyńska et al. 2019) in comparison to healthy subjects. Because the expression of glycosyltransferases is the main factor influencing cell glycome (Moremen et al. 2018), the reduced level of *FUT8*, *MGAT5*, and *ST6Gal1* gene transcripts suggests the lower core fucosylation and β1,6-branching of complex-type *N*-glycans, and α2,6-sialylation of glycoconjugates in the activated pool of CD4+ T helper cells from AITD patients (Korta et al. 2019; Ząbczyńska et al. 2019). Galectin-glycoprotein lattice, dependent on GlcNAc β1,6-branching of *N*-glycans on T cell receptor (TCR), regulates TCR clustering within the cell membrane and its recruitment to the site of antigen presentation by APCs (Demetriou et al. 2001; Dennis et al. 2009). Because of the reduced *MGAT5* expression and β1,6-branching of *N*-oligosaccharides on TCR decreases a threshold of TCR activation which may contribute to autoimmune disease development (Demetriou et al. 2001), the significantly lower *MGAT5* expression in CD4+ T cells from HT and GD (Korta et al. 2019; Ząbczyńska et al. 2019) may be important in AITD pathology. The lowered surface fucosylation on T cell affects also its activation as it was also shown using 2-fluorofucose (2FF), a fucose analog. 2FF-treated T cells with the decreased fucosylation were more easily activated due to a reduced avidity of galectin-3 to bind down-fucosylated glycoproteins and a lowered TCR threshold which increased TCR signaling (Field et al. 2016).

The diverse composition of serum and immune cell glycome, including the reduced core fucosylation of IgG and other serum proteins, is one of the alterations that occur in thyroid autoimmunity. The functional aspect of these changes is the primary goal of further research.

10.4 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting connective tissue. SLE involves different organs and displays many clinical presentations. SLE is more prevalent in women than men with a 9:1 ratio (D'Cruz et al. 2007). Primary serum biomarkers of SLE are autoantibodies against ANA such as double-stranded DNA (dsDNA), ribonucleoproteins (RNPs), and Smith (Sm). Autoantibodies associated with their targets form the immune complexes (IC) which accumulate in target organs. The deposition of IC in tissues initiates the inflammatory response and leads to tissue injury. SLE predominantly affects skin, joints, central nervous system, and kidneys (Dema and Charles 2014, 2016).

As in the case of RA, studies on the role of glycosylation in the pathogenesis of SLE and the search for glyco-markers of the disease are partially focused on the analysis of IgG glycosylation in patients. The studies of Frano Vučković and coworkers described significant differences in IgG glycome between SLE patients and healthy controls, showing decreased galactosylation, sialylation, and core fucosylation together with increased content of bisecting GlcNAc (Vučkovïc et al. 2015). Moreover, the authors found that the majority of glycan structures were strongly associated with disease status. This study performed on a large population of 475 SLE patients of different ethnicity, partially resembles the findings presented earlier on RA patients, suggesting again the possible role of agalactosylation of IgG in autoimmunity. On the other hand, the authors postulate that significant changes in sialylation, core fucose, and bisecting GlcNAc seem to be more specific for SLE. Other studies underline the possible importance of exposed core fucosyl residues of native circulating IgG complexes from active SLE patients in interactions with lectin receptors of immunological importance (Sjöwall et al. 2015). However, in these studies, the exact IgG glycome was not analyzed, but only the interactions of these complexes with fucose-binding lectins were investigated.

Another part of the studies on the impact of glycosylation on SLE pathogenesis concerns the possible importance of glycan modifications in the mechanisms of autoimmunity. Surface glycosylation of the resting and activated T cells from SLE patients and healthy donors was investigated using a set of lectins. The comparison of resting

T cells from SLE and healthy donors demonstrated an increase of core fucosylation of diantennary *N*-glycans, the higher content of high-mannose structures, and GlcNAc in hybrid-type oligosaccharides/sialylation of complex multi-antennary *N*-glycans using LCA, Concanavalin lectin (ConA), and wheat germ agglutinin (WGA) respectively. The activated T cells from SLE showed a significant decrease of galectin-1 binding activity corresponding to the lower content of *N*-acetyllactosamine (LacNAc, Galβ1,4GlcNAc disaccharide unit) on T lymphocyte glycoproteins. The reduced binding of galectin-1 was also related to the enhanced sialylation of SLE *N*-glycans, because the forming of the galectin-glycoprotein lattice on neuraminidase-treated T cells was restored. The upregulated sialylation of activated T cells from SLE patients was accompanied by the overexpression of *ST3Gal6* gene encoding α2,3-sialyltransferase which is responsible for adding α2,3-Sia (Szabó et al. 2019).

The mouse model with the null allele of α-mannosidase II (αMII), an enzyme important for the synthesis of complex-type *N*-glycans, showed a decreased level of complex structures in the liver, kidney, brain, heart, and spleen. A lower level of complex-type oligosaccharides was accompanied by symptoms similar to SLE. 80% of 6-month age mice showed antibody deposition in kidney glomeruli. The frequency of lymphocytic infiltration in the liver, kidney, and lungs was higher in mutant mice. Adding to that, their serum IgG, IgM, and IgA level was elevated with normal frequencies of CD4+ and CD8+ T cells as well as CD220+ B cells at the same time. Over 60% of αMII deficient mice with hematuria have a positive titer of ANA recognizing histone, Sm antigen and double- and single-stranded DNA, the typical SLE autoantigens (Chui et al. 2001). The continuation of the study on bone marrow graft mice model using both wild type and null mice as donor and recipient showed that elevated autoantibody levels, IgG, and complement C3 deposition in kidneys and kidney inflammation are associated only with αMII deficient mice recipient. This suggests that mechanisms triggering αMII-dependent autoimmune responses are not related to abnormalities in hematopoiesis (Chui et al. 2001). Another group of authors investigated kidney glomeruli mesangial cells, which can produce cytokines including monocyte chemoattractant protein-1 (MCP-1) responsible for recruitment and activation of innate immune cells (macrophages) in the response to the stimuli in circulation during the filtration process. Isolated glomeruli treated with sera of αMII deficient mice revealed an increase in MCP-1 production in comparison to stimulation with wild type mouse sera. The response was inhibited by the treatment with mannose analog and mannose lectin binding inhibitor. The author suggests that hybrid-type *N*-glycans present in the sera of αMII deficient mice are responsible for mesangial cell activation by a mannose-dependent binding mechanism (Green et al. 2007).

CD4+ cells isolated from peripheral blood of SLE patients manifest the higher FUT8 activity in comparison with healthy individuals' cells. Taking into consideration that the percentage of activated CD4+ cells increase in SLE, the higher FUT8 efficiency of total CD4+ may be due to CD4+ activation. To confirm the hypothesis that core fucosylation affects the CD4+ activation Wei Liang and co-workers used the mouse model of $Fut8^{-/-}$ and $Fut8^{+/+}$ with induced experimental autoimmune encephalomyelitis (EAE). A lack of Fut8 enzyme reduced symptoms of EAE. To analyze if TCR fucosylation influences CD4+ activation the isolated cells from $Fut8^{-/-}$ and $Fut8^{+/+}$ mice were analyzed in terms of ZAP70 phosphorylation (pZAP70), a marker of TCR signaling transduction. The levels of pZAP70 in $Fut8^{-/-}$ CD4+ T cells were decreased in comparison to $Fut8^{+/+}$ CD4+ T cells. Additionally, the impact of core fucosylation on TCR activation via interaction with MHCII presenting peptide was determined. T cell activation with peptide presented by MHCII was significantly lower in $Fut8^{-/-}$ CD4+ T cells. The authors suggested that core-fucosylation is crucial for TCR–MHC-II interaction in CD4+ T cell activation and the high level of core fucosylation may serve as a glycobiomarker in SLE (Liang et al. 2018).

Finally, there are some data concerning the impact of SLE-associated autoantibodies glycosylation on the disease pathogenesis. The studies

of Iryna Magorivska and co-workers showed that the affinity-purified anti-histone autoantibodies from SLE patients displayed a lower sialylation status than total IgG (Magorivska et al. 2016). Moreover, the authors found that in functional *ex-vivo* studies on phagocytosis of post-apoptotic cells, secondarily necrotic cell-derived material (SNEC), the non-sialylated anti-SNEC autoantibodies directed the SNEC preferentially into polymorphonuclear cells (PMNs) without inducing anti-inflammatory cytokine response. These findings suggest the possible important role of autoantibody sialylation in the modulation of the immune response in SLE patients (Magorivska et al. 2016).

10.5 Inflammatory Bowel Disease

IBD are a group of chronic inflammatory diseases of the gastrointestinal tract containing Crohn's disease (CD) and ulcerative colitis (UC). Inflammation and ulceration in IBD are mainly caused by the infiltration of neutrophils and macrophages into the intestinal mucosa (Guan 2019). One of the widely accepted hypotheses of IBD pathogenesis is that IBD is caused by an autoimmune response to self-antigens and normal intestinal flora. Among well-described antibodies characteristic for IBD are perinuclear anti-neutrophil cytoplasmatic antibodies (pANCA), anti-*Saccharomyces cerevisiae* antibodies (ASCA), and anti-pancreatic antibodies (Vermeulen et al. 2011).

The expression of β1,6-GlcNAc branched *N*-glycans in lamina propria CD3+ infiltrated cells was checked using *Phaseolus vulgaris* lectin (PHA-L). The analysis showed a significant decrease of β1,6-GlcNAc structures in colonic biopsies of UC patients in comparison to healthy control. To evaluate the presence of β1,6-GlcNAc on TCR receptor, lamina propria lymphocytes (LPL) were isolated. Lectin blotting showed a significant decrease of PHA-L binding to TCR. Moreover, the expression of β1,6-GlcNAc decreased with the severity of the disease. Results of gene expression of *MGAT5* in LPL are in the accordance with lectin analysis and showed a significant decrease in *MGAT5* expression in UC patients in comparison to control donors (Dias et al. 2014). CD3+ cells isolated from colonic biopsies and peripheral blood were supplemented *ex vivo* with GlcNAc. GlcNAc supplementation caused an increased β1,6-GlcNAc branching of *N*-glycans on colonic T cells as well as PBMC from UC patients. The observation was common both for CD4+ and CD8+ cells, however, GlcNAc supplementation does not affect CD4+/CD8+ ratio. Interestingly, T cells from healthy donors and patients with inactive disease did not show higher levels of branched glycans after GlcNAc treatment. GlcNAc supplementation of *ex vivo* activated T cells from UC resulted in the significant reduction of their proliferative response to anti-CD3/CD28 mAb stimulation and the decreased synthesis of pro-inflammatory cytokines typical for UC. Results obtained in *ex vivo* tests were confirmed on two mouse models of colitis (Dias et al. 2018). These studies revealed the same tendency to reduction of branched *N*-glycans in TCR as described in UC patients. Moreover, chemical induction of colitis in *Mgat5* null or deficient mice resulted in their higher susceptibility to severe forms of colitis in comparison to WT mice. Treatment of colitis *Mgat5*$^{+/-}$ mice with GlcNAc caused suppression of colitis severity. Mouse model showed that the induction of colitis is accompanied by reduced expression of branched *N*-glycans in LPL. These findings underline the possible importance of β1,6-GlcNAc branching in IBD pathogenesis. Other studies also explored another glycosylation modification. Using mouse model and patients' specimens the role of core fucosylation in UC was analyzed (Fujii et al. 2016). *Fut8*$^{-/-}$ and wild type *Fut8*$^{+/+}$ mice were subjected to UC induction. Wild type mice with chemically induced colitis showed an increase of core fucosylation level in splenocytes in comparison to non-colitis mice. Histologic analysis showed more severe colonic inflammation in *Fut8*$^{+/+}$ mice than in *Fut8*$^{-/-}$ mice. Inflamed intestinal mucosa in IBD patients showed higher *FUT8* mRNA expression in comparison to the non-inflamed tissue. Expression of core fucose was increased on T cells from colitis mice compared to mice without colitis as well as on inflamed intestine mucosa from IBD patients, compared to non-inflamed tissues or tissues from control patients. Mouse *Fut8*$^{-/-}$ model showed a less

severe symptom of colitis which is associated with the reduced production of cytokines by Th (Fujii et al. 2016). All the described data suggest that core fucosylation by modulation of T cell signaling can play an important role in colitis induction.

Similarly, as in other autoinflammatory diseases, IgG glycosylation in IBD patients' sera was also analyzed. The studies of Irena Trbojević Akmačić and co-workers describe the glycomic analysis of IgG from 507 UC and 287 CD patients in comparison to healthy controls (Trbojevic Akmacic et al. 2015). They found, that in both UC and CD, IgG galactosylation was significantly reduced. CD was also characterized by significant down-regulation of sialylated *N*-glycans. These observations again underline the increased inflammatory potential of IgG in the course of autoimmune diseases. Moreover, studies by Shinichiro Shinzaki and co-workers conclude that G0F/G2F glycans ratio can be a potential diagnostic biomarker of the disease activity in both IBDs (Shinzaki et al. 2008). Finally, there are also some limited studies concerning other serum-derived biomarker glycosylation in IBD, mainly the acute-phase proteins (Theodoratou et al. 2014). However, it is not yet possible to draw any unequivocal conclusions about the prognostic potential of their glycan modifications.

10.6 Multiple Sclerosis

MS is a central nervous system inflammatory disease manifesting in demyelination, inflammation, axonal loss, and gliosis. The infiltration of immune cells and the appearance of inflammatory mediators leads to tissue lesions (Mirshafiey and Kianiaslani 2013) manifested clinically with motor, sensory, visual, and autonomic systems functional deficits (Compston and Coles 2008). Among MS autoantigens the best described are myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and myelin-associated basic oligodendrocytic protein (MOG) (Cavallo 2020).

Glycosylation studies of MS are still not very numerous. There are of course some data describing IgG glycosylation patterns associated with MS. The studies by Ana Cvetko and co-workers show that core fucosylation and abundance of high-mannose structures were the most altered IgG glycosylation traits in MS patients compared to controls (Cvetko et al. 2020). Adding to that, these studies compared the plasma protein *N*-glycomes and the shift towards increased complexity of glycans (elevation of highly branched, tri- and tetra-antennary, sialylated structures) were found in MS patients. Another study also showed that *N*-glycosylation of IgG1 is different between cerebrospinal fluid (CSF) and serum of MS patients and that CSF-derived IgG1 *N*-glycosylation changes comparing to controls (Wuhrer et al. 2015). The main IgG modifications in CSF from MS patients determined in this study were elevated bisecting GlcNAc and reduced galactosylation. Another interesting glycosylation study on MS regards MOG autoantigen recognized by autoantibodies of patients with inflammatory central nervous system disease. MOG protein has one *N*-glycosylation site in a position N31. The influence of *N*-glycosylation on anti-MOG reactivity was analyzed by non-glycosylated MOG mutant using sera from anti-MOG positive patients with inflammatory CNS diseases. It was shown that the lack of *N*-glycan does not influence MOG conformation. Among 27 tested patient sera 60% reacted differently with at least one of the mutants in comparison to wild type MOG (Marti Fernandez et al. 2019). Finally, some data suggested that alterations in the expression of *N*-glycan branching enzymes (such as GnT-IV and GnT-V) in T cells are associated with MS and that increased *N*-glycan branching on T cells can reduce the risk of MS (Chien et al. 2018).

10.7 Conclusions

Searching for changes in glycan composition characteristic for autoimmune diseases is one of the most significant goals of recent studies from borderline in research areas of glycobiology and immunology. The examples of the obtained results provided in this chapter, summarized in Fig. 10.1, indicate the considerable importance of glycosylation in autoimmunity. Most of the data on glycosylation in autoimmune diseases was

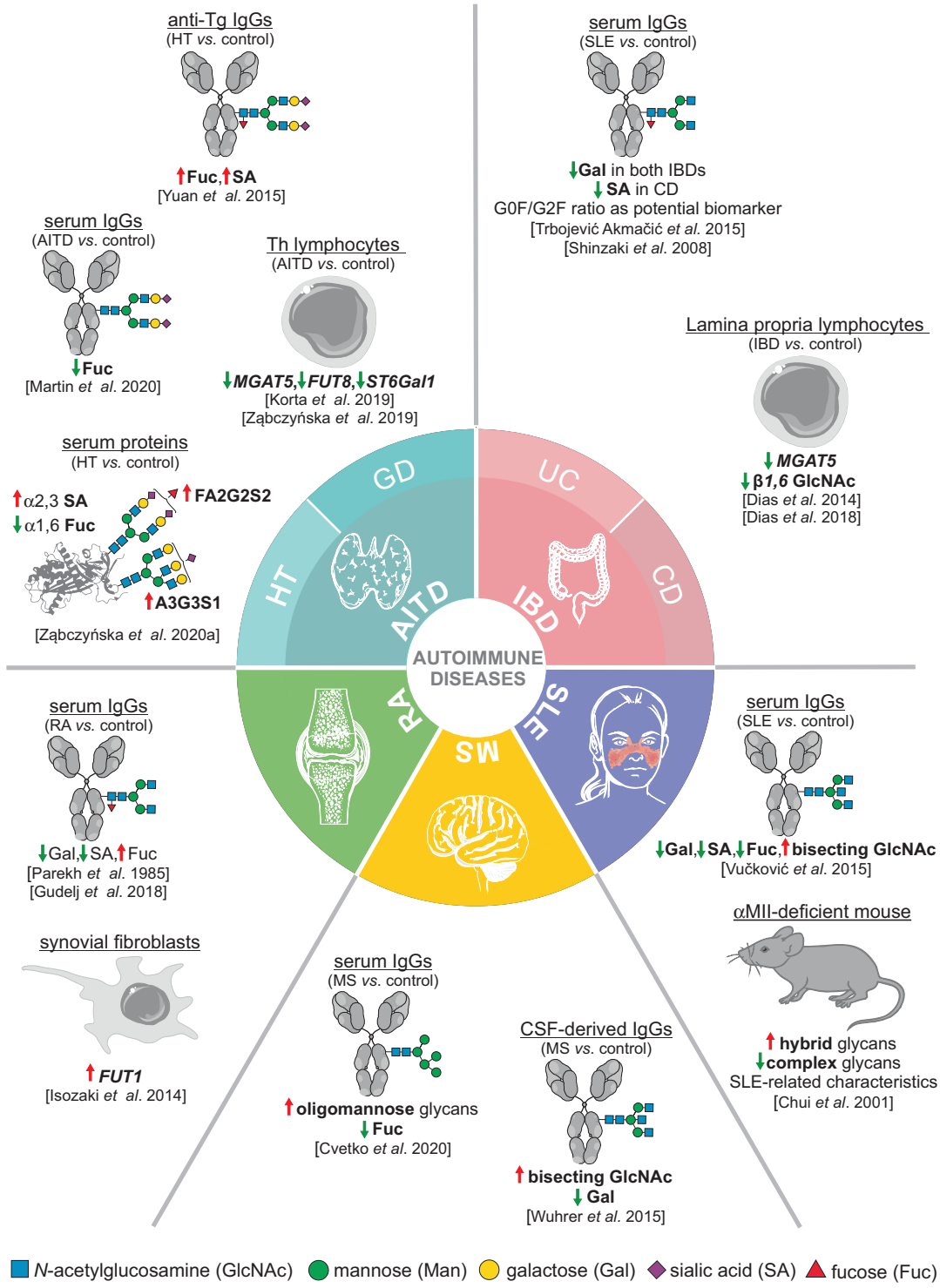

Fig. 10.1 Glycosylation changes in serum and cellular proteins in autoimmune diseases. *AITD* autoimmune thyroid disease, *αMII* alpha-mannosidase II, *CD* Crohn's disease, *CSF* cerebrospinal fluid, *FUT1/8* genes encoded fucosyltransferases 1/8, *GD* Graves' disease, *HT* Hashimoto's thyroiditis, *IBD* inflammatory bowel disease, *IgG* immunoglobulin G, *MGAT5* gene encoding β1,6-*N*-acetylglucosaminyltransferase V, *MS* multiple sclerosis, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *ST6Gal1* gene encoding α2,6-sialyltransferase, *Tg* thyroglobulin, *Th* T helper, *UC* ulcerative colitis. The schemes from the website https://smart.servier.com (SMART Sevier Medical Art, published by Les Laboratoires Servier) were applied to prepare this figure

obtained for serum IgG, mainly because of the key role of this protein in the induction of autoimmunity and also due to the availability of IgG, and the relatively easy method of its isolation. Knowing the versatile roles of oligosaccharides relevant at protein and cell levels, it is not surprising that detected structural changes of glycans have a profound effect on autoimmunity, especially on the pro-inflammatory activity of immune cells and soluble proteins.

Compliance with Ethical Standards

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Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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The Role of Glycosylation in Infectious Diseases

11

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Abstract

Glycosylation plays an important role in infectious diseases. Many important interactions between pathogens and hosts involve their carbohydrate structures (glycans). Glycan interactions can mediate adhesion, recognition, invasion, and immune evasion of pathogens. To date, changes in many protein *N*/*O*-linked glycosylation have been identified as biomarkers for the development of infectious diseases and cancers. In this review, we will discuss the principal findings and the roles of glycosylation of both pathogens and host cells in the context of human important infectious diseases. Understanding the role and mechanism of glycan–lectin interaction between pathogens and hosts may create a new paradigm for discovering novel glycan-based therapies that can lead to eradication or functional cure of pathogens infection.

Keywords

Glycosylation · Virus · Bacteria · Glycan–lectin interaction · Infection · Immune evasion

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11.1 Introduction

Glycobiology is an emerging field focused on defining the structures and functional roles of carbohydrate structures, called glycans, in biological systems. These glycan structures, composed of branched chains of monosaccharides, are added to a wide variety of biological molecules (such as proteins and lipids) through a biological process called glycosylation (Semchenko et al. 2012; Tao et al. 2008; Eichler 2019; Stowell et al. 2014; Waespy et al. 2015). Glycosylation alters not only protein/lipid structure but also their function. Glycan is not just a biomarker of biological functions but actually plays critical roles in modulating immune responses. The specific structure of a glycan allows it to bind to a specific type of glycan-binding proteins called lectins, leading to activation of downstream signaling pathways of cells.

In eukaryotes, more than 50% of proteins are glycosylated (Crocker and Feizi 1996; Parodi 2000; Munkley 2017). Glycosylation can affect the development of many immune-related diseases, including infections and tumors, leading to the emergence of sugar-chain-specific changes. Changes in these glycans not only affect the course of the disease, but also can be used as biomarkers for diagnosis of diseases.

In this review, we will discuss the role of glycosylation in the context of human important

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viral and bacterial infectious diseases. We will also highlight the prospects for the lectin/inhibitor of glycosylation-based therapies to treatment of infectious diseases. Understanding glycan–lectin interaction between pathogen and host will help to realize the goals of personalized medicine and its impact on human health.

11.2 Glycosylation in Viral Diseases

Attachment of viruses to cell-surface receptors is the initial step in infection. Over half of all mammalian virus families recognize host cell surface glycans as receptors. Meanwhile, the virus itself lacks glycosylation modification systems; therefore, the glycosylation of virus is modified by the synthetic system in host cell. From a viral perspective, viral glycosylation plays an important role in viral protein folding, viral infection invasion, participation in recognition of host cell receptors, and participation in the body's immune escape against viruses.

Here, we summarize some of the important role and the mechanism of glycans and glycolipids from both viral envelope glycoproteins and host cell-surface as shown in Table 11.1. The characteristics of interaction between virus and host, based on either the viral glycan binding to host cell lectin or viral lectin binding to glycan receptor of host cell, are summarized in Table 11.1.

11.2.1 Human Immunodeficiency Virus (HIV)

Acquired immune deficiency syndrome (AIDS) is an infectious disease caused by human immunodeficiency virus (HIV) and has done great harm to human beings. HIV-1 envelop (Env) presents between 18 and 33 glycans per gp120 monomer, with a median of 25 glycan sites on gp120 and 4 glycan sites on the gp41 subunit. These glycans, mainly high-mannose-type glycosides, and mixed structures such as glycosides and sialic acid (Mathys et al. 2014; Kumar et al. 2011) (Table 11.2), bind to CD4, chemokine receptors (Losman et al. 2001), dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN), or macrophage mannose receptor (MR) on the host cell and play important roles in HIV-1 entry (Wolk and Schreiber 2006; Vigerust and Shepherd 2007). DC-SIGN recognition of HIV high mannose glycans mediates HIV capture by dendritic cells (DCs), which can subsequently lead to $CD4^+$ T cell trans-infection. DC-SIGN plays a key role in the dissemination of HIV-1 by DCs (Geijtenbeek and van Kooyk 2003). The binding of heavily glycosylated HIV Envto certain C-type lectin receptors (CLRs) on different antigen presenting cell (APC) populations might influence T-cell priming and/or B-cell activation (Heß et al. 2019; Yolitz et al. 2018; Watanabe et al. 2019), also shield this important Ag from recognition by neutralizing Abs and cytolytic $CD8^+$T cells. *N*-glycans of HIV-1 gp120 are critical determinants that can profoundly influence $CD4^+$ T cell recognition (Li et al. 2008). The inhibitor of glucosidase I and II, *N*-butyldeoxynojirimycin (N-DNJ), can inhibit HIV from entering host cells in vitro and affect the synthesis of viral envelope components (Fischer et al. 1996). The possible mechanism of this effect is to reduce the binding of HIV envelope protein gp120 to CD4 molecules on the surface of T lymphocytes (Papandréou et al. 2002). HIV-1 particles are also captured by CD169, an I-type lectin, whose expression on DCs is enhanced upon maturation with LPS (Akiyama et al. 2015). Human serum L-ficolin or mannose-binding lectin (MBL) can bind to HIV gp120, blocks HIV infection, and induces complement-dependent cytotoxicity (Luo et al. 2016).

HIV infection also causes certain host IgG glycomic alterations, including hypo-sialylation and agalactosylation (lack of galactose) (Vadrevu et al. 2018). Lower levels of galactosylation in HIV+ individuals compared to healthy controls are most pronounced in the IgG1 subclass (Moore et al. 2005). A rapid secretion of galectin-9 has been described after HIV infection, and it was shown that galectin-9 suppresses B cell receptor

signaling (Cao et al. 2018). How glycan–lectin interactions impact these important T cell functions during HIV infection is not yet completely clear. Clarifying the role these interactions play during HIV infection can provide insights that may lead to the development of novel therapies.

11.2.2 Hepatitis C Virus (HCV)

HCV is the most thoroughly studied of Flaviviruses. More than 180 million people worldwide have developed chronic hepatitis C infection (Waziry et al. 2017; Pradat et al. 2018). HCV infection causes chronic liver diseases, liver fibrosis, and even hepatocellular carcinoma (HCC). HCV has two enveloped heavily glycosylated proteins with up to 5 sites on E1 and up to 11 sites on E2 modified by *N*-linked glycans (Goffard and Dubuisson 2003; Lavie et al. 2018; Min et al. 2017) (Tables 11.1, 11.2). HCV E1/ E2 heterodimer envelope glycoproteins play an essential role in virus entry and assembly, and show poor immunogenicity due to their high glycosylation. *N*-glycosylation deletion mutations on the envelope glycoproteins E1/E2 could enhance antigen-presenting activity and cellular and neutralizing antibody responses (Goffard and Dubuisson 2003; Lavie et al. 2018; Ren et al. 2016; Xiang et al. 2017; Helle et al. 2010; Li et al. 2007; Liu et al. 2007), suggesting the lack of protein/peptide glycosylation is a feature that most likely favors effective recognition by T cells. The *N*-glycosides on HCV envelope glycoprotein may inhibit the adaptive immune response by affecting some antigen epitopes, thus inducing the immune escape of the virus (Li et al. 2007; Liu et al. 2007).

Despite enormous variation in HCV envelope genetically (about 35%), sites occupied by glycans are highly conserved (Goffard and Dubuisson 2003). The glycosylation sites of HCV envelope glycoproteins E1 and E2 may be directly involved in the binding of the virus to cellular receptors. This binding has an important effect on the invasion of virions (Choukhi et al. 1998; Trombetta and Helenius 1998; Helle and Dubuisson 2008; Goffard et al. 2005). After the loss of glycosylation, the protein cannot be transported to the membrane or secreted outside the cell, resulting in a significant decrease in the efficiency of virus particles invading cells (Lavie et al. 2018). Human serum L-ficolin inhibits the binding of HCV envelope glycoprotein E1/E2 to its cell receptors, the low-density lipoprotein receptor (LDLR) and the scavenger receptor-BI (SRBI), blocking the invasion of HCV on susceptible cells (Zhao et al. 2014).

In addition to the effect of HCV glycans themselves on infection and immune response, HCV infection can also lead to the alteration of the glycosylation of host hepatocytes with the increase of cell proliferation and drug resistance (Li et al. 2019). The fucosylation, sialylation, and complex *N*-glycan levels were significantly increased in HCV-infected Huh7.5.1 cells compared to uninfected cells (Xiang et al. 2017). It was also determined that fucosylated Annexin A2 (ANXA2) and fucosylated heat shock protein 90 beta family member 1 (HSP90B1) were significantly increased in HCV-infected cells (Xiang et al. 2017). Increased fucosyltransferase 8 (FUT8) by HCV infection induced 5-FU drug resistance of Huh7.5.1 cells through NF-κB-MRP1/P-gp signaling (Li et al. 2019).

11.2.3 Hepatitis B Virus (HBV)

HBV, belonging to Hepadnaviridae, is considered a serious risk factor for chronic inflammation, cirrhosis, and HCC (Ganem and Prince 2004). *N*-glycosylation of the main hydrophilic region of HBV surface antigen (HBsAg) is essential for the secretion of virus particles (Ito et al. 2010) (Table 11.1). The formation of HBV envelope was inhibited after the glycosylation of the three glycoproteins L, M, and S antigens of the viral envelope was lost (Lu et al. 1995). Core fucosylation modified by FUT8 was also upregulated by HBV infection, similar phenomenon as for HCV. High FUT8 expression often indicates a poor prognosis for HBV-related liver cancer (Takamatsu et al. 2016). Core fucosylation is an important glycosylation modification in the process of HBV infection of liver cancer cells

Table 11.1 Role of the glycosylation of virus and host in viral diseases

Glycan-lectin	Virus	Roles of glycosylation in viral invasion, immunity, and biology	Ref
Viral glycans bind to host lectins	**Retroviridae** (e.g., human immunodeficiency virus, (HIV))	The gp120 contains up to 20–30 glycosylation sites, mainly high-mannose-type glycosides, and mixed structures such as glycosides and sialic acid, bind to CD4, chemokine receptors, DC-SIGN(R), Langerin, L-selectin, and mannose receptor (MR) on the host cells	Wolk and Schreiber (2006); Vigerust and Shepherd (2007)
		N-glycans in the V1/V2 loop of 15 variants of HIV-1 NL4-3 gp120 reduce viral infectivity and sensitivity to serum antibodies	Wolk and Schreiber (2006)
		DC-SIGN plays a key-role in the dissemination of HIV-1 by DCs	Geijtenbeek and van Kooyk (2003)
		HIV-1 particles captured by CD169, an I-type lectin, whose expression on DCs is enhanced upon maturation with LPS	Akiyama et al. (2015)
		L-ficolin/MBL binds to HIV gp120, blocks HIV infection, and induces complement-dependent cytotoxicity	Luo et al. (2016)
		HIV infection also causes certain IgG hypo-sialylation and agalactosylation (lack of galactose)	Vadrevu et al. (2018)
	Flaviviridae (e.g., hepatitis C virus (HCV))	HCV contains heavily high-mannose *N*-glycosylated with up to 5 sites on E1 and up to 11 sites on E2 envelope glycoproteins. *N*-glycans of HCV E1 and E2 envelope proteins mediate the immune escape of the virus	Goffard and Dubuisson (2003); Lavie et al. (2018); Min et al. (2017); Ren et al. (2016)
		The levels of fucosylation, sialylation, and complex *N*-glycans are significantly increased in HCV-infected cells	Xiang et al. (2017)
		L-ficolin inhibits the binding of HCV envelope glycoprotein E1/E2 to its cell receptors LDLR and SRBI, blocking the invasion of HCV on susceptible cells, while ApoE3 competitively blocks L-ficolin's effect and promotes HCV infection.	Zhao et al. (2014)
	Hepadnaviridae (e.g., hepatitis B virus (HBV))	*N*-glycosylation of the main hydrophilic region of HBV surface antigen (HBsAg) is essential for the secretion of virus particles	Ito et al. (2010)
		High expression of FUT8 induced by HBV suggests poor prognosis of HBV-related liver cancer	Takamatsu et al. (2016)
		Serum L-ficolin is closely related to the degree of inflammation and the efficacy of anti-HBV therapy	Chen et al. (2015)

Viral lectins bind to glycan receptors of host cells	**Orthomyxoviridae** (e.g., influenza virus)	HA of human viruses exhibits a preference for glycan receptors with terminal NeuAca2-6Gal linkages on host cells; HA of avian viruses recognizes receptors with the NeuAcα2-3Gal linkage	de Graaf and Fouchier (2014); Paulson and de Vries (2013)
	Coronaviridae (e.g., SARS)	The zoonotic MERS strain spike recognizes dipeptidyl peptidase 4 as a receptor, and NeuAc2-3Gal-containing glycans as co-receptors	Li et al. (2017); Raj et al. (2013)
		Human β-coronaviruses (OC43, HKU1) spike recognizes 9-*O*-AcNeuAc as a receptor on epithelial cells of the respiratory tract	Huang et al. (2015); Langereis et al. (2015)
	Paramyxoviridae (e.g., Newcastle disease virus (NDV))	Paramyxoviruses recognize glycans with the terminal NeuAcα2-3Gal linkage on host cells	Alymova et al. (2012); Amonsen et al. (2007)
	Picornaviridae (e.g., coxsackie A24)	Human enteroviruses bind glycan receptors with terminal NeuAcα2-6Gal and/or NeuAcα2-3Gal linkages	Zocher et al. (2014)
		Porcine enterovirus binds ganglioside receptor such as GD1a	Kim et al. (2016)
	Polyomaviridae (e.g., HPyV-1)	Virus binds preferentially to sialoglycans of host cells	Maginnis et al. (2015); O'Hara et al. (2014)
	Reoviridae (e.g., rotavirus)	Most animal rotaviruses bind terminal NeuAcα2-3Gal/GalNAc sequences as found on GM3 or GD1a gangliosides	Haselhorst et al. (2009)
	Adenoviridae (e.g., human adenoviruses (HAdV))	HAdV-D binds NeuAcα2-3Gal-terminated glycans	Arnberg et al. (2002)
		HAdV-52 preferentially recognizes polysialic acid (NeuAcα2-8NeuAcα2-8) sequences	Lenman et al. (2018)

Flaviviridae: positive, single-stranded, enveloped RNA viruses, including hepatitis C virus, yellow fever, dengue fever, Japanese encephalitis, West Nile viruses, and zika virus
Retroviridae: these viruses encode a reverse transcriptase enzyme that converts the RNA genome into DNA during the retroviral life cycle, which then becomes integrated into the host genome, such as human immunodeficiency virus (HIV)
Hepadnaviridae: enveloped viruses with T = 4 icosahedral capsids. Genomes are relaxed circles of partially double-stranded DNA, such as hepatitis B virus (HBV)
Orthomyxoviridae: membrane-enveloped, segmented negative-sense RNA viruses divided into seven genera known as influenza virus A, B, C, and D, isavirus, quaranjavirus, and thoratovirus
Paramyxoviruses: membrane-enveloped viruses with a continuous, negative-sense single-stranded RNA genome, including Newcastle disease virus (NDV), Sendai, mumps, and parainfluenza viruses 1,3,5
Coronaviruses (CoVs): membrane-enveloped, single-stranded positive-sense RNA viruses
Picornaviruses: non-enveloped viruses containing a single positive strand RNA genome (7.5 kb) within a 30 nm icosahedral capsid including human (e.g., coxsackie A24) enteroviruses
Polyomaviruses: non-enveloped icosahedral viruses with double-stranded DNA genomes, including a-polyomavirus, b-polyomavirus, g-polyomavirus, and d-polyomavirus
Reoviridae: a large family of double-stranded RNA viruses, including rotavirus and orthoreovirus
Adenoviruses: non-enveloped icosahedral viruses with double-stranded DNA genomes

Table 11.2 Several important viral envelope glycans structure

Virus	Glycosylated protein	Glycosylation site	Glycan structure	Ref
HCV	E1, E2	4–11	High mannose, hybrid	Goffard and Dubuisson (2003)
IAV	HA, NA	5–11	High mannose, GlcNAc	Kim et al. (2013); Tate et al. (2014)
HIV	Envelope gp120	20–30	High mannose, hybrid, sialic acid	Mathys et al. (2014); Kumar et al. (2011)
SARS-CoV-2	S1, S2	22	High mannose, hybrid, complex, sialic acid	Watanabe et al. (2020)
Ebola virus	GP1, 2	8–15	High mannose, hybrid, sialic acid	Collar et al. (2017)

through its receptor-mediated endocytosis, so down-regulation of core fucosylation can be regarded as a new target for both HBV and HCV treatment (Li et al. 2019; Takamatsu et al. 2016). Human serum L-ficolin is closely related to the degree of inflammation and the efficacy of anti-HBV therapy (Chen et al. 2015).

11.2.4 Influenza Virus

Influenza A virus (IAV), belonging to Orthomyxoviridae, causes pandemics worldwide each year and occasional pandemics. IAV carries two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), of which HA is the main surface glycoprotein on the influenza envelope, mediating attachment and entry into the host through interaction with the salivary acid receptor. After translation, HA relies on glycosylation of host cells, which is essential for proper folding and transport of molecules during infection (Daniels et al. 2003). The envelope protein HA has 5–11 glycosylation sites, and most of them are usually located in the spherical head of the molecule (Kim et al. 2013; Tate et al. 2014) (Table 11.2).

In most cases of virus invasion into host cells, IAV utilizes sialic acid (NeuAc-containing glycans) as host cell receptors (Zhang et al. 2015). Human influenza viruses exhibit a preference for glycan receptors with terminal NeuAcα2-6Gal linkages (Table 11.2), while avian viruses recognize receptors with the NeuAcα2-3Gal linkage, commonly referred to as "avian-type" and "human-type" receptor specificity (de Graaf and Fouchier 2014; Paulson and de Vries 2013).

Surfactant protein D (SP-D) and L-ficolin are the primary innate inhibitors of IAV in the lung (Goh et al. 2013; Pan et al. 2012). SP-D/L-ficolin-HA interaction could significantly block viral infection of target cells and viral aggregation and neutralization (Goh et al. 2013). These studies provide the insight of the innate host defense lectins inhibiting their viral glycoprotein target.

11.2.5 Coronaviruses

Coronaviruses (CoVs) infect humans and animals and cause a variety of diseases, including respiratory, intestinal, kidney, and neurological diseases, including the severe acute respiratory syndrome coronavirus (SARS-CoV) (de Wit et al. 2016; Lau et al. 2005), Middle East respiratory syndrome coronavirus (MERS-CoV), and the newly emerged SARS-CoV-2, termed coronavirus disease 19 (COVID-19).

CoVs typically contain two surface glycoproteins: the spike protein that is primarily responsible for attachment and membrane-fusion (Table 11.1) and a hemagglutinin-esterase that can also participate in attachment (Song et al. 2018). The outer membrane spike glycoproteins of coronaviruses are known for its glycosylation (Xiong et al. 2018) and interact with host cell targets (e.g., angiotensin-converting enzyme 2

(ACE2), CD26, Ezrin, cyclophilin, and other cell adhesion factors). The zoonotic MERS strain spike recognizes dipeptidyl peptidase 4 (DPP4) as a primary receptor and NeuAc2-3Gal-containing glycans as co-receptors (Li et al. 2017; Raj et al. 2013) (Table 11.1). Recent studies have reported that human ACE2 is also the entry receptor of SARS-CoV-2 and that the serine protease TMPRSS2 is also important for the activation of SARS-CoV-2 S (Hoffmann et al. 2020). Human β-coronaviruses (OC43, HKU1) spike recognizes 9-*O*-AcNeuAc as a receptor on epithelial cells of the respiratory tract (Huang et al. 2015; Langereis et al. 2015).

11.2.6 Other Viruses

In addition, paramyxoviruses (e.g., Newcastle disease virus (NDV)) recognize glycans with the terminal NeuAcα2-3Gal linkage on host cells (Alymova et al. 2012; Amonsen et al. 2007). Human enteroviruses (e.g., coxsackie A24) bind glycan receptors with terminal NeuAcα2-6Gal and/or NeuAcα2-3Gal linkages (Zocher et al. 2014), and porcine enterovirus binds ganglioside receptor such as GD1a (Kim et al. 2016). Polyomaviridae (e.g., HPyV-1) bind preferentially to sialoglycans of host cells (Maginnis et al. 2015; O'Hara et al. 2014). Most animal rotaviruses bind terminal NeuAcα2-3Gal/GalNAc sequences as found on GM3 or GD1a gangliosides (Haselhorst et al. 2009). Adenoviridae (e.g., human adenoviruses (HAdV)) HAdV-D binds NeuAcα2-3Gal-terminated glycans and HAdV-52 preferentially recognizes polysialic acid (NeuAcα2-8NeuAcα2-8) sequences (Arnberg et al. 2002; Lenman et al. 2018) (Table 11.1).

However, the host cell receptors for several important viral enveloped carbohydrate structures (e.g., high mannose, hybrid, and complex types) of IAV (Kim et al. 2013; Tate et al. 2014), SARS-CoV-2 (Watanabe et al. 2020), and Ebola virus (Collar et al. 2017) (Table 11.2) are unknown yet. Further studies will help to reveal the mechanism of viral infection and pathogenicity and provide potential therapeutic strategies for these viruses based on glycan–lectin interactions.

11.3 Glycosylation in Bacterial Diseases

Next we discuss the roles of glycosylation in the pathogenesis and immune responses of human important pathogenic bacteria. The glycan interactions between bacterial pathogens and their hosts involved in adhesion, immune escape, and host colonization are shown in Table 11.3.

11.3.1 *Mycobacterium tuberculosis* (*M. tb*)

Tuberculosis (TB) is a disease caused by *M. tb* and remains a major global health problem. *M. tb* still likely infects approximately one-quarter of the world's population and is one of the top ten causes of death and the leading cause of death from a single infectious agent worldwide. Up to now, effective means for TB diagnosis, especially for bacillus-negative (Bn) TB laboratory diagnosis, are urgently needed. The only TB vaccine currently available is the attenuated strain of *Mycobacterium bovis*, known as Bacillus Calmette and Guérin (BCG), which has limited efficacy in adults (Shah et al. 2008).

The polysaccharides or liposaccharides on the walls of *M. tb* play important roles in the regulation of different immune responses and the activation or inhibition of different immune cascade reactions induced by *M. tb* (Karakousis et al. 2004). Among them, the immunoregulatory role of lipoarabinomannan (LM) (Kumar et al. 2011) has attracted much attention (Lawn 2012). Its precursors, including LM, phosphatidyl-myo-inositol mannosides (PIMs), have been shown to have potential regulatory effects in vitro experiments on immune cells (Dao et al. 2004; Gilleron et al. 2003). Mannose-capped lipoarabinomannan (ManLAM) is a major cell wall lipoglycan

and an important immunomodulatory component of mycobacteria (Kang et al. 2005; Sun et al. 2016; Tang et al. 2017; Gringhuis et al. 2009; Yonekawa et al. 2014; Yuan et al. 2019; Torrelles and Schlesinger 2010) (Table 11.3). Bacterial ManLAM can also be secreted and recognized by macrophages and DCs via pattern recognition receptors, including MR, Toll-like receptor 2 (TLR2), DC-SIGN, CD1d, sphingosine-1-phosphate receptor 1 (S1P1), Dectin-2, and CD44, and triggers several cell signaling pathways (Sun et al. 2016; Yonekawa et al. 2014; Pan et al. 2014; Osanya et al. 2011; Zajonc et al. 2006). It is well known that ManLAM inhibits phagosome maturation of macrophages, DC maturation, and $CD4^+$ T cell activation (Osanya et al. 2011; Fratti et al. 2003; Mahon et al. 2012). Anti-ManLAM antibody treatment and anti-ManLAM aptamer treatment decrease bacterial loads and dissemination, prolong survival, and lead to better disease outcomes in an animal model of TB (Pan et al. 2014; Hamasur et al. 2004). ManLAM can promote the polarization of *M. tb*-responsive $CD4^+$ T cells to Th2 type and reduce the Th1-type immune response by inhibiting the antigen presentation function of DCs (Pan et al. 2014). ManLAM aptamer can enhance BCG immunogenicity and has a good therapeutic effect in mouse and rhesus *M. tb* infection models (Pan et al. 2014).

By using high-performance liquid chromatography (HPLC) and mass spectrometry, a recent study showed that significantly higher agalactosylated (G0) vs monogalactosylated (G1) and digalactosylated (G2) *N*-glycans of IgG were found in TB patients including the smear negative TB patients than in healthy donors. *M. tb* infection caused the increase of IgG–Gal ratio G0/(G1 + G2 × 2) in *M. tb* infection mouse model (Liu et al. 2020). Quantitative analysis of serum-based IgGGal ratio G0/ (G1 + G2 × 2) could be used for TB auxiliary diagnosis (Liu et al. 2020).

In addition, using lectin microarrays it was shown that infection of virulent *M. tb* H37Rv, but not attenuated *M. tb* H37R*a*, induces increased Galβ1–3GalNAc-glycosylated CD44 on macrophage (Tang et al. 2017). Addition of ABA lectin blocked the attachment/engulfment of *M. tb* H37Rv to macrophage. ABA directly binds with Galβ1–3GalNAc-glycosylated CD44 on macrophage and inhibits *M. tb* ManLAM binding to glycosylated CD44. These studies will help to reveal the mechanism of pathogenicity and virulence of *M. tb* from a new perspective and provide a potential new diagnostic and therapeutic strategy for tuberculosis based on glycopatterns, ABA, and its ligand Galβ1–3GalNAc-glycosylated CD44 target molecule on macrophage.

11.3.2 *Helicobacter pylori* (*H. pylori*)

The Gram-negative bacterium *H. pylori* colonizes the gastric mucosa of over half the global human population and increases the risk of gastric cancer. The adherence of *H. pylori* is mediated by bacterial BabA adhesin protein which is specific for the Lewis blood group antigens sialyl Lewis X (sLeX) and sialyl Lewis A on gastric epithelial cells (Ilver et al. 1998) (Table 11.3). Low levels of sialic acids are expressed in the gastric lining of healthy individuals. Sialic acid expression is increased in the stomach lining after *H. pylori* has established a chronic infection (Magalhães et al. 2015), demonstrating that *H. pylori* influences the host gastric glycome. Recently, another adhesin, LabA (also known as LacdiNAc-binding adhesin) of *H. pylori*, was found to bind LacdiNAc structures (GalNAcβ1-4GlcNAc) conjugated to MUC5AC mucins in the gastric mucosal layer. This adhesin may have a role in persistence by retaining *H. pylori* in the mucosal layer.

11.3.3 *Streptococcus pyogenes*

S. pyogenes (group A Streptococcus (GAS)) causes a range of human diseases, including acute pharyngitis and skin diseases. M protein, a major virulence factor of GAS and a trigger of the autoimmune reaction that leads to rheumatic fever (Fischetti 1989), interacts with glycosami-

Table 11.3 Glycan interactions between bacterial pathogens and their hosts

Bacteria	Roles of glycosylation in bacterial infection and immunity	Ref
M. tb	ManLAM of *M. tb* binds to MR of macrophage	Kang et al. (2005); Sun et al. (2016)
	ManLAM of *M. tb* binds to Galβ1-3NAc on CD44 glycoprotein of macrophage	Sun et al. (2016); Tang et al. (2017)
	ManLAM of *M. tb* binds to DC-SIGN on dendritic cells to promote IL-10 secretion	Gringhuis et al. (2009)
	ManLAM of *M. tb* binds to Dectin-2 receptors on dendritic cells, and promotes the release and secretion of TNFs, IL-6, and IL-10	Yonekawa et al. (2014)
	ManLAM of *M. tb* binding with TLR2 induces B cells to secrete IL-10	Yuan et al. (2019)
	Significantly more agalactosylated (G0) vs monogalactosylated (G1) and digalactosylated (G2) *N*-glycans of IgG in TB patients, including smear-negative TB patients, than in HDs	Liu et al. (2020)
	Virulent *M. tb* H37Rv infection induces increased Galβ1-3GalNAc-glycosylated CD44 on macrophage. Addition of ABA blocked the attachment/engulfment of *M. tb* H37Rv to macrophage	Tang et al. (2017)
H. pylori	BabA of *H. pylori* is specific for the Lewis blood group antigens sialyl Lewis X (sLeX) and sialyl Lewis A on gastric epithelial cells	Ilver et al. (1998)
GAS	The M protein of GAS interacts with the blood group H antigen of blood cells	De Oliveira et al. (2017)
	The molecular weight of the HA expressed on the GAS polysaccharide capsule has roles in immune evasion	Schommer et al. (2014)
GBS	The polysaccharide capsules of GBS interact with Siglec-9 as a method of immune evasion	Carlin et al. (2009)
E. coli	Type 1 fimbrin (FimH) of *E. coli* binds oligomannose on the host uroepithelium during urinary tract infections	Sokurenko et al. (1998)
	The glycosylation of a host protein NleB1 by *E. coli* avoids caspase 8 activation and as such prevents host cell death receptor apoptosis	Pearson et al. (2013)
N. gonorrhoeae	The pili of *N. gonorrhoeae* can be glycosylated with the disaccharide Gal (α1-3) diNAcBac	Jennings et al. (1998)
N. meningitidis	The increased capsular polysaccharide expression in *N. meningitidis* leads to impairment of the alternative complement pathway and escape	Uria et al. (2008)
	The *N. meningitidis* serogroup B repeating α2-8Neu5Ac binds with host sialylglycoconjugates	Giuliani et al. (2006)
	3'-sialyllacto-*N*-neotetraose made by *N. meningitidis* interacting with Thomsen-Friedenreich tumor antigen on the host cell surface	Mubaiwa et al. (2017)
C. jejuni	The *N*-linked protein glycosylation of *C. jejuni* protects bacterial proteins from cleavage by host gut proteases	Alemka et al. (2013)
	The asialo-G_{M1} terminal structure of *C. jejuni* interacts with the host glycan, blood group B antigen	Day et al. (2015)
	The *C. jejuni* LoS mimics the carbohydrate Gm1 ganglioside on host cells	Janssen et al. (2008)

noglycans for adherence to host cell (Frick et al. 2003). A recent study by glycan microarray screening demonstrated that M protein had a preference for H blood antigen over A and B blood antigens which are highly expressed on oral epithelial cells and in mucosal fluid (Table 11.3). The ability of M protein to bind certain classes of glycans of H blood antigen may have a major role in host tissue tropism and disease outcome (Maamary et al. 2012; De Oliveira et al. 2017).

GAS expresses a capsule comprising hyaluronan, which mimics host glycosaminoglycan in the skin. The length of the chains of hyaluronan influences the survival of GAS in skin and deep tissue infections through interaction with the cell surface receptor CD44 (Everest-Dass et al. 2012). The molecular weight of hyaluronan attached to the GAS capsular surface can influence uptake by macrophages suggests that glycans on the bacterial surface assist in immune system evasion (Schommer et al. 2014).

11.3.4 *Escherichia coli* (*E. coli*)

Pathogenic *E. coli* is an important human pathogen that can cause a range of diseases from enteritis to urinary tract infections (UTIs) and meningitis; uropathogenic *E. coli* (UPEC) is a major cause of UTIs. *E. coli* uses several different types of pili to adhere to host surfaces, and the binding specificities of these pili determine host and tissue tropism. For example, type 1 pili of *E. coli* carry the lectin FimH, which mediates binding to mannose on the surface of host intestine cells of healthy humans (Sokurenko et al. 1998) (Table 11.3), whereas FimH from *E. coli* isolated from UTIs preferentially binds oligomannose host glycans (Karakousis et al. 2004). This preference for oligomannose by UPEC has been used as the basis for rational drug design of C1-modified α-mannoside-based FimH antagonists. A class of mannosides, biphenyl α-d-mannopyranosides, has shown potential as UTI therapeutics.

Another *E. coli* pilus adhesin, UclD, has lectin function. The UclD lectin domain binds to colonic epithelial cells, and this interaction was inhibited by pretreatment of *O*-glycosidase to remove *O*-linked glycans, suggesting UclD interacts with one or more *O*-linked glycans on host cells (Spaulding et al. 2017).

Some other glycan interaction between bacteria and host, such as the polysaccharide capsules of GBS (Carlin et al. 2009), bacterial glycosylated surface protein (Pearson et al. 2013; Jennings et al. 1998; Uria et al. 2008; Giuliani et al. 2006; Mubaiwa et al. 2017; Alemka et al. 2013; Day et al. 2015), or bacterial mimic of host carbohydrate Gm1 ganglioside (Janssen et al. 2008), has also been implicated causing immune evasion and impairment of immune response (Table 11.3).

11.4 Host Lectins and Infectious Diseases

Lectins, glycan-binding proteins, from host cells have been reported to play important roles in the immune system and have evolved to initiate the host immune response to glycans produced by multiple pathogens, such as the C-type lectin DC-SIGN (Ito et al. 2010; Gringhuis et al. 2009; de Witte et al. 2007), the MBL (Xiang et al. 2017) and the ficolins. Several important host lectins which recognize viral and bacterial glycans are summarized in Table 11.4. Here we focus to discuss the recent discoveries about the ficolins during viral and bacterial infection.

Ficolins are the recently identified serum complement lectins, with a structure similar to MBL and lung surfactant protein SP-A and SP-D. Ficolins belong to family of collectins that activate the lectin pathway of complement. In humans, three ficolins have been characterized: Ficolin-1 (M-Ficolin), Ficolin-2 (L-ficolin), and Ficolin-3 (H-ficolin). In mice, two types of ficolins, Ficolin-A and Ficolin-B, have been identified. Based on the structural and functional properties and phylogenetic analysis, mouse Ficolin-A resembles human Ficolin-2.

Table 11.4 Host lectins recognize viral and bacterial glycans

Lectin types	Related viruses	Glycan structure	Mechanism	Ref
Ficolins	HCV/HBV/HIV/IAV	High mannose	Eliminates HCV by binding E1/E2 *N*-glycans; Binds to viral glycan and blocks viral infection	Luo et al. (2016); Ito et al. (2010); Chen et al. (2015); Ding et al. (2017); Kilpatrick et al. (2009); Ren et al. (2014)
SP-A and SP-D	IAV	High mannose, GlcNAc	Inhibits influenza virus infection of A549	Al-Qahtani et al. (2019)
MBL	HIV	High mannose, hybrid, sialic acid	Participates in HIV recognition through high mannose structure	Luo et al. (2016)
DC-SIGN, MBL, MR, Langerin	HIV-1	High mannose, Hybrid	Viral antigen presentation through high mannose structure	Hart et al. (2002)
DC-SIGN	HIV-1/HCV	High mannose	Viral antigen presentation through high mannose structure	de Witte et al. (2007)

Ficolin-A/2 is mainly synthesized in the liver and present in the circulation, and has a lectin-like activity in the recognition of *N*-acetylglucosamine (GlcNAc), lipopolysaccharides (LPS), β-1,3-D-glucan, lipoteichoic acid and various acetylated compounds. Ficolins are able to recognize pathogen-associated molecular patterns (PAMPs) which are carbohydrate molecules on the surface of pathogens, and of apoptotic, necrotic, and malignant cells. Upon binding to their specific PAMPs, ficolins may trigger activation of the immune system by initiating activation of complement via the lectin pathway, opsonophagocytosis, or by stimulating secretion of the inflammatory cytokines interferon (IFN)-γ, interleukin (IL)-17, IL-6, and tumor necrosis factor (TNF)-α, and production of nitric oxide (NO) by macrophages, thus limiting the infection and concurrently orchestrating the subsequent adaptive immune response (Ren et al. 2014). Recently, a number of reports have shown that dysfunction or abnormal expression of ficolins may play crucial roles in viral and bacterial diseases and in inflammation.

Here we sum up the reports on the roles of ficolins in the infectious diseases, and provide insight into ficolins as novel innate immune therapeutic options to treat these diseases.

L-ficolin has been reported to bind to several important viral *N*-glycans from HCV, HIV, IAV, and HBV and blocks their viral infection to target cells (Luo et al. 2016; Ito et al. 2010; Chen et al. 2015; Liu et al. 2009; Luo et al. 2013). L-ficolin binds to *N*-glycans of the envelope proteins E1/E2 on the surface of HCV, triggering C4 deposition to activate the complement lectin pathway in HCV-infected hepatocytes (Liu et al. 2009). L-ficolin competitively inhibits the binding of HCV envelope glycoprotein to its cell receptors LDLR and SRBI, blocking the invasion of HCV on susceptible cells, while ApoE3 in HCV particles can inhibit the anti-HCV infection effect of L-ficolin by competitively binding HCV-E2, promoting HCV infection, and mediating viral immune escape (Ito et al. 2010). L-ficolin also binds to the HIV envelope protein gp120, blocks HIV infection, and induces complement-dependent cytotoxicity. L-ficolin prevents HIV-1

from entering and infecting target cells (TZM-b1 and MT-2 cells) in a dose-dependent manner (Geijtenbeek and van Kooyk 2003). L-ficolin could recognize and bind HA and NA and inhibit influenza virus infection, and these interactions could be competitively inhibited by addition of GlcNAc.

In human important bacterial diseases, L-ficolin has also been reported to bind to glycolipid ManLAM on the surface of *M. tb* to inhibit the invasion of *M. tb* target cells. Serum levels of L-ficolin were much lower in TB and tumor patients than in healthy controls (Luo et al. 2013; Ding et al. 2017). In vitro experiments demonstrated that L-ficolin binds to the surface glycolipid of *H37Rv* and blocks *H37Rv* infection in human lung A549 cells. L-ficolin plays an anti-tuberculosis role by activating the JNK phosphorylation pathway, up-regulating the secretion of cytokines IFN-g, IL-17, TNF-α, and the active molecule NO (Luo et al. 2013). L-ficolin deficiency is associated with susceptibility to human infection of TB and it could be a novel immunotherapeutic molecule against TB in the future.

L-ficolin also binds to Gram-negative bacteria, such as the rough type of *Salmonella typhimurium* (*S. typhimurium*) (TV119) and *P. aeruginosa* (Kilpatrick et al. 2009; Taira et al. 2000). Related studies have reported that L-ficolin protein binds to *S. typhimurium* strains with exposed GlcNAc at the non-reducing end of the polysaccharide, which enhances the surface GlcNAc clearance of pathogens and acts as an opsonin (Taira et al. 2000). In addition, human ficolins are associated with Gram-positive bacterial infectious diseases. The combination of L-ficolin with type III group B *streptococcus* or *S. aureus* leads to the activation of the lectin pathway (Lynch et al. 2004). L-ficolin has been shown to activate the lectin complement pathway by binding carbohydrates on the bacterial surface and enhance the opsonization activity of polymorphonuclear neutrophils (Aoyagi et al. 2005).

Besides L-ficolin, some recent studies also showed that both M-ficolin and H-ficolin also had affinity for GlcNAc in Gram-negative bacteria (Akaiwa et al. 1999; Liu et al. 2005). M-ficolin has been reported to bind to several novel glycoproteins on *S. aureus* containing GlcNAc, GalNAc, and NeuAc-*N*-acetyllactosamine (Frederiksen et al. 2005). H-Ficolin is combined with *Aerococcus viridans*, which is the only Gram-positive bacterium it can bind (Tsujimura et al. 2001).

Some other human lectins, such as SP-A/D (Al-Qahtani et al. 2019), DC-SIGN, MBL, MR, and Langerin (Hart et al. 2002), also play important roles by blocking viral infection (Al-Qahtani et al. 2019) and inhibition of viral antigen presentation (Hart et al. 2002) (Table 11.4). These data suggest that lectin-based therapies could be used for the treatment of viral and bacterial diseases.

11.5 Conclusions

Glycosylation is a complex biological process that defines and regulates several key physiological processes in organisms. It is a double-edged sword, which not only helps the host to recognize and eliminate the virus, but can also be a tool for the pathogens to escape the host immunity. The functional research on glycosylation has just begun, and the research on the relationship between some diseases and protein glycosylation is not mature enough. We have described the major roles of glycosylation in the development of important viral and bacterial diseases. Information about the potential therapeutic significance of glycoproteins and lectins was also mentioned.

Currently, we will need further work to reveal more detailed knowledge about the nature of the interaction between glycans and receptors. Illustrating the infection in the process of protein glycosylation modification mechanism will help to discover new molecular targets of glycosylation modification related to infection of important pathogens and immune evasion. Creating new anti-infection strategies targeting abnormal glycosylation modification will also help to develop new glycosylation inhibitors as anti-pathogen agents. Development of glycan-based strategies, for example, anti-glycan vaccines, glycan lectin interaction blockers,

glycan-specific monoclonal antibodies, and glycan-coated nanoparticles, will provide new directions for improving the diagnosis or treatment methods used to treat human infectious diseases.

Compliance with Ethical Standards

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The Importance of Glycosylation in COVID-19 Infection

12

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Abstract

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is currently one of the major health problems worldwide. SARS-CoV-2 survival and virulence are shown to be impacted by glycans, covalently attached to proteins in a process of glycosylation, making glycans an area of interest in SARS-CoV-2 biology and COVID-19 infection. The SARS-CoV-2 uses its highly glycosylated spike (S) glycoproteins to bind to the cell surface receptor angiotensin-converting enzyme 2 (ACE2) glycoprotein and facilitate host cell entry. Viral glycosylation has wide-ranging roles in viral pathobiology, including mediating protein folding and stability, immune evasion, host receptor attachment, and cell entry. Modification of SARS-CoV-2 envelope membrane with glycans is important in host immune recognition and interaction between S and ACE2 glycoproteins. On the other hand, immunoglobulin G, a key molecule in immune response, shows a distinct glycosylation profile in COVID-19 infection and with increased disease severity. Hence, further studies on the role of glycosylation in SARS-CoV-2 infectivity and COVID-19 infection are needed for its successful prevention and treatment. This chapter focuses on recent findings on the importance of glycosylation in COVID-19 infection.

Keywords

COVID-19 · Glycosylation · SARS-CoV-2 · S glycoprotein · Viral infection

12.1 Introduction

The recent emergence and rapid global spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is currently one of the major health problems in the world. SARS-CoV-2 causes a respiratory illness named coronavirus disease 2019 (COVID-19). SARS-CoV-2 is closely related to SARS (retrospectively named SARS-CoV-1) and Middle East respiratory syndrome (MERS) coronaviruses (CoVs), causing zoonotic epidemics and local outbreaks in 2003 and 2012, respectively (De Wit et al. 2016). All

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three highly pathogenic CoVs originated from bats. Following a likely zoonotic spillover, human-to-human transmission events were confirmed with clinical presentations ranging from no symptoms to mild fever, cough, and dyspnea to cytokine storm, respiratory failure, and death. Although risk factor for susceptibility to COVID-19 has been evaluated in several studies, there are currently limited known risk factors. Some of them are age, environmental factors, chronic illness, unhealthy lifestyle, and ABO blood group (Kifer et al. 2021; Lauc and Sinclair 2020; Zheng et al. 2020; Zhou et al. 2020a). Also, several research groups analyzed genetic data to understand the links between human genetic variation and COVID-19 susceptibility (Asselta et al. 2020; Renieri et al. 2020; Cao et al. 2020; Pairo-Castineira et al. 2020; Stawiski et al. 2020).

12.1.1 Glycosylation

Covalent attachment of glycans (oligosaccharides) is an enzyme-directed process, termed glycosylation. Glycans are one of the four basic building blocks of life and are mostly bound to proteins or lipids in the endoplasmic reticulum (ER) and the Golgi apparatus of the cellular secretory pathway (Varki et al. 2009). Proteins are typically modified by *N*- or *O*-glycosylation. *N*-glycosylation refers to the attachment of *N*-acetylglucosamine (GlcNAc) to the nitrogen atom of an Asn side chain by a β−1 *N*-linkage. These Asn-linked glycoconjugates contain a mannose ($Man_3GlcNAc_2$) core, to which a variable number of other monosaccharides can be added or removed. These additions include GlcNAcylation, galactosylation, sialylation, and fucosylation, and they determine whether the final structure is classed as a high-mannose, a hybrid, or a complex *N*-glycan. *N*-glycosylation depends on the formation of a lipid precursor in which GlcNAc and Man form a branched carbohydrate structure on the cytoplasmic side of the ER (Varki et al. 2015). Following completion, an oligosaccharyltransferase adds the carbohydrate chain to a protein at an Asn-X-Ser or Asn-X-Thr amino acid sequence, where X may be any of the amino acids except Pro. The nascent carbohydrate–protein conjugate undergoes further processing in the ER, which usually involves the removal of the glucose residues as part of a quality-control process. The structure then moves to the Golgi apparatus for maturation (Varki et al. 2015).

Glycosylation can also occur on amino acids with functional hydroxyl groups Ser or Thr via *N*-acetylgalactosamine (GalNAc), or much less frequently via GlcNAc, Man, or fucose (Fuc) (Varki et al. 2017). Glycoproteins containing GalNAc-linked glycans, often called mucin-type *O*-glycans, are the most abundant extracellular glycoproteins, including mucins (Vasudevan and Haltiwanger 2014; Bennett et al. 2012). Mucins interface between epithelial cells and the external mucosal surfaces of the body. They are characterized by numerous tandem repeats of Pro, Ser, and Thr, which create many sites for *O*-glycosylation. Extended *O*-glycan cores on these sites create a gel-like substance thought to protect both the glycoproteins and cellular surfaces from external stress, microbial infection, and self-recognition by the immune system. *O*-glycans contain cores 1–4, terminal GalNAc (Tn), and sialyl-Tn antigens and are integral, along with other *O*-linked glycoconjugates, to the classification of blood group antigens. Mucin-type *O*-glycan synthesis is initiated by polypeptide GalNAc transferases (GalNAc-Ts), where these GalNAc-Ts differ in their specificity for amino acid motifs (Varki et al. 2017). The addition of glycans to the initial GalNAc results in a diverse set of carbohydrate structures that are often highly clustered on certain glycoproteins, e.g., mucins and human immunoglobulin A1 (IgA1). These sugars are added as the protein moves through the Golgi compartments and, in contrast to *N*-glycan synthesis, there is no preprocessing and post-processing of existing sugar structures. Instead, diverse glycosyltransferases modify the existing *O*-glycan structure with galactose, GlcNAc, sialic acid (Sia), and Fuc (Varki et al. 2017).

Unlike proteins, glycans are not directly encoded in the genome and are therefore secondary gene products. At least 10% of the human genome is responsible for the production of

enzymes and transporters required in the biosynthesis of glycan structures (Lauc et al. 2014). Glycoproteins participate in numerous molecular processes including cell adhesion, protein folding, receptor activation, and endocytosis (Ohtsubo and Marth 2006). Also, glycosylation is a key mechanism that regulates the function of immunoglobulins (Igs), with multiple systemic repercussions to the immune system (Gudelj et al. 2018; Lauc et al. 2016), indicating the importance of this process.

Glycans play a major role in the immune system, particularly in modifying the inflammatory response in terms of maintaining the function of the mucosal barrier, distinguishing "one's own" from "other's" and the behavior of immune cells (Lauc et al. 2016). Human cells are covered with glycocalyx, a thick layer of glycans bound to membrane proteins and lipids. The glycocalyx is at least 10 and sometimes 1000 times thicker than the cell membrane itself. This envelope is a cellular fingerprint, the way the body distinguishes "its" from "another's." If a cell with a different, unknown envelope is found in our body (e.g., an infectious organism, transplanted tissue, but also its own diseased cells), the body will soon start its defense mechanism against such a cell (Lauc et al. 2016). Glycan diversity represents one of the main defenses against pathogens. Moreover, protein glycosylation plays a crucial role in viral pathogenesis (Watanabe et al. 2019, 2020a), as shown by the characteristically thick *N*-glycan coating of the viral fusion proteins (Stewart-Jones et al. 2016; Crispin et al. 2018). The biological roles of the *N*-glycans expressed on the surface of viral envelope glycoproteins are very diverse and all are linked to their nature (Watanabe et al. 2019).

Any glycan bound to a particular protein, synthesized on one glycosylation site, can exist in multiple structural variants, the phenomenon termed microheterogeneity. Thus, a particular protein, encoded by a single gene, may have different glycoforms, which make up a separate molecular entity. The purpose of microheterogeneity is thought to be to generate biodiversity and defend against pathogens and parasites (Varki et al. 2009).

12.2 SARS-CoV-2 Structure and Cell Entry

12.2.1 Virion Protein Structure

CoVs are the largest group of viruses belonging to the Nidovirales order, in which all viruses are enveloped, non-segmented positive-sense RNA viruses. CoV particles contain four main structural proteins, named the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Maier et al. 2015).

The most abounded structural protein is the M protein, which directs protein–protein interactions required for the assembly of CoVs. Also, M protein is responsible for the shape of the virion, but M protein alone is not sufficient for virion formation. When M protein is expressed along with E protein, which is present in small quantities, they produce CoV envelopes (Bos et al. 1996).

It is unknown how E protein assists M protein in the assembly of the virion, but several possibilities have been suggested. Boscarino et al. suggested that E protein prevents the aggregation of M protein (Boscarino et al. 2008), although others suggested that the E protein may have a separate role in promoting viral release by altering the host secretory pathway (Ye and Hogue 2007). Other work has indicated that E protein has a role in inducing membrane curvature (Schoeman and Fielding 2019). SARS-CoV-2 E protein has been predicted to contain two potential *N*-glycosylation sites, N48 and N66 (Duart et al. 2020), although site N48 is not expected to be occupied due to the proximity of this glycosylation acceptor site to the membrane if the neighboring hydrophobic region is recognized as a transmembrane by a translocon (Duart et al. 2020). Further studies on the occupancy of these predicted *N*-glycosylation sites are needed.

The N protein is heavily glycosylated, and it is the only protein present in the nucleocapsid. It is composed of two separate domains, an N-terminal domain (NTD) and a C-terminal domain (CTD), both capable of binding RNA in vitro. Phosphorylation has been suggested to trigger a structural change enhancing the affinity for viral

RNA. N protein binds the viral genome in a beads-on-a-string type conformation. N protein also binds a key component of the replicase complex (Hurst et al. 2009, 2013), and the M protein (Sturman et al. 1980). These protein interactions help to package viral genome into viral particles.

The pathogenic SARS-CoV-2 enters human target cells via its viral transmembrane S glycoprotein. S glycoprotein is synthesized as a single 1273 amino acid polypeptide chain on the rough ER (Duan et al. 2020). During synthesis, co-translationally, *N*-linked high-mannose oligosaccharide side chains are added (Helenius and Aebi 2001; Aebi 2013). After synthesis, the S glycoprotein monomers trimerize, facilitating the transport from the ER to the Golgi complex. Each monomer can be divided into three main topological domains, namely the head, stalk, and cytoplasmic tail (CT) (Casalino et al. 2020). Once in the Golgi complex, most of the high-mannose oligosaccharide side chains are modified to more complex forms (Zhang and Wang 2016; Schwarz and Aebi 2011), and *O*-linked oligosaccharide side chains are also added (Andersen et al. 2020; Joshi et al. 2018). In the trans-Golgi, the SARS-CoV-2 S glycoprotein is proteolytically cleaved by cellular furin or furin-like proteases at the S1/S2 cleavage site (Hoffmann et al. 2020a; Coutard et al. 2020). Cleavage at the S1/S2 site yields a surface subunit S1 (harboring the receptor-binding domain, RBD) and S2 (containing the membrane fusion domains) (Mounir and Talbot 1993; Abraham et al. 1990). The S1 subunit facilitates attachment of the virus while the S2 subunit facilitates fusion of the viral and human cellular membranes (Zhou et al. 2020b; Hoffmann et al. 2020b; Walls et al. 2020). Furin-like cleavage is essential for the S-protein-mediated cell–cell fusion and viral infectivity and is required for efficient SARS-CoV-2 infection of airway epithelial cells (Bestle et al. 2020) and human lung cells (Hoffmann et al. 2020a). Like in other CoVs, S glycoprotein mediates attachment to the host receptors, using angiotensin-converting enzyme 2 (ACE2) receptor and/or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin 1 (DC-SIGNR) as the dominant mechanism of cell entry (Hoffmann et al. 2020b; Walls et al. 2020; Wan et al. 2020a; Shang et al. 2020; Lu et al. 2020; Letko et al. 2020).

The hemagglutinin-esterase (HE) is the fifth structural protein, which is present in a subset of β-CoVs. The protein acts as a hemagglutinin (HA), binds to specific receptors that possess terminal *N*-acetylneuraminic acid (Neu5Ac, Sia), particularly on epithelial cells and mucins of human respiratory tracts, and enhance virus spread through the mucosa (Lang et al. 2020). SARS-CoV-2 HE acts as the classical glycan-binding lectin and receptor-degrading enzyme. Most β-CoVs recognize 9-*O*-acetyl-Sia but switch to recognizing the 4-*O*-acetyl-Sia form during the evolution of CoVs reflecting viral evolutionary adaptation to host Sia-containing glycans. Type I HE is specific for the 9-*O*-acetyl-Sia and type II HE is specific for 4-*O*-acetyl-Sia (Kim 2020).

12.2.2 SARS-CoV-2 Glycosylation

Viral glycosylation has wide-ranging roles in viral pathobiology, including mediating protein folding and stability and shaping viral tropism (Watanabe et al. 2020b). Numerous enveloped viral pathogens have evolved to take advantage of host cell glycosylation processes and decorate the surface of their proteins with "self" glycan moieties shielding them from immune system recognition and targeting. Viral glycosylation has been investigated in various envelope glycoproteins, including CoV S glycoprotein (Walls et al. 2016; Xiong et al. 2018; Ritchie et al. 2010), HA glycoprotein of influenza virus (Wanzeck et al. 2011; Lee et al. 2014; Kobayashi and Suzuki 2012), human immunodeficiency virus-1 (HIV-1) (Struwe et al. 2018; Panico et al. 2016; Cao et al. 2018), Ebola virus glycoprotein (Lee et al. 2008), and E glycoprotein of dengue, Zika, and other flaviviruses (Mondotte et al. 2007; Fontes-Garfias et al. 2017). Furthermore, analysis of viral glycosylation across different families has revealed significant variation in the density, structure, and conservation of glycans. High viral glycan density and local protein archi-

tecture can sterically impair the glycan maturation pathway, resulting in the presence of oligomannose-type glycans that can be a reporter of native-like protein architecture (Behrens et al. 2017).

Epitope masking by glycosylation has been observed on SARS-CoV S protein (Walls et al. 2016, 2019), as well as in HIV-1 Env (Jardine et al. 2013) and influenza HA proteins (Xu et al. 2010; Wei et al. 2010). Ritchie et al. observed that SARS-CoV S protein has 30% of oligomannose-type glycans (Ritchie et al. 2010), while Watanabe et al. show that sites N234 and N709 of SARS-CoV-2 S protein are mainly occupied by oligomannose-type glycans (Watanabe et al. 2020b). Also, the predominant oligomannose-type glycan observed across the protein is $Man_5GlcNAc_2$ (except for *N*-glycosylation site N234). This indicates that these *N*-glycosylation sites are more accessible to α-1,2-mannosidase, than are for *N*-acetylglucosaminyltransferase I (GlcNAcT-I), which allows further processing to hybrid- and complex-type glycans in the Golgi apparatus. The overall oligomannose-type glycan proportion of around 30% is low (Ritchie et al. 2010) compared to the other viruses such as HIV-1, influenza, and Lassa (more than 50%) where sites are dominated by $Man_9GlcNAc_2$ glycan (Watanabe et al. 2020b; Struwe et al. 2018; Behrens et al. 2017). Moreover, compositional analysis of the glycan shield of alpha- and delta-CoVs, expressed in *Drosophila* S2 cells, also displayed a wide variety of oligomannose-type glycans (Walls et al. 2016; Xiong et al. 2018). Less dense glycosylation across the surface indicates that antibodies (Abs) can target the protein surface. The role of glycosylation in camouflaging immunogenic protein epitopes has been studied for other CoVs (Watanabe et al. 2020b; Walls et al. 2019; Yang et al. 2020). Notably, there have been no observed mutations in *N*-glycosylation sites of SARS-CoV-2 so far (Watanabe et al. 2020b).

Site-specific analysis of *N*- and *O*-glycosylation of SARS-CoV-2 S protein provides an understanding of the viral structure, crucial for the identification of immunogens for vaccine design (Hargett and Renfrow 2019). *N*-glycans on S protein play important roles in proper protein folding and priming by host proteases (Walls et al. 2020). As glycans can shield the amino acid residues from Ab recognition, glycosylation can enable CoV to evade both the innate and adaptive immune responses (Walls et al. 2019, 2020). The glycosylation pattern of S protein should be considered regarding chemical properties, steric hindrances, and even as a potential target for a mutation in the future.

S protein is highly glycosylated, with 22 predicted *N*-glycosylation sites and 3 *O*-glycosylation sites on S1 and S2 subunits (Andersen et al. 2020; Uslupehlivan and Şener 2020). Several groups have reported on their occupancy and glycan type but with some conflicting results emphasizing the importance of the context in which the S protein is expressed and purified before analysis. While Watanabe et al. have found that all 22 *N*-linked glycosylation sites are occupied most of the time (Watanabe et al. 2020b), Walls et al. provided evidence for the existence of 16 *N*-glycans on 22 potential sites in the SARS-CoV-2 S protein by cryogenic electron microscopy (Walls et al. 2020). On the other hand, Shajahan et al. observed partial *N*-glycan occupancy on 17 out of 22 *N*-glycosylation sites and 5 unoccupied *N*-glycosylation sites on recombinant SARS-CoV-2 S1 and S2 subunits individually expressed in human HEK293 cells. They observed both high-mannose and complex-type glycans and found no hybrid-type *N*-glycans, highlighting divergent processing of the SARS-CoV-2 S protein glycans compared to host glycoproteins (Shajahan et al. 2020; Loke et al. 2016). They found highly processed sialylated complex-type glycans at RBD *N*-glycosylation sites that can act as a determinant in viral binding with human ACE2 (hACE2) receptors. Confirming findings by Watanabe et al., Shajahan et al. also observed $Man_5GlcNAc_2$ as a predominant structure across all S1 sites (Watanabe et al. 2020b; Shajahan et al. 2020). However, they observed significantly unoccupied peptides on eight *N*-glycosylation sites, five on S1 subunit and three on S2 subunit for which *N*-glycosylation detection was ambiguous. However, the authors cautioned that these

findings should be validated with further studies (Shajahan et al. 2020).

In another study by Zhao et al. (2020a) a trimer-stabilized, soluble variant of the SARS-CoV-2 S protein was expressed and purified from HEK293 cells, resulting in the quantification of 49 *N*-glycans (28% of high-mannose type, 17% of the hybrid type, and 55% of the complex type). A high degree of core fucosylated, bisected, and Lac diNAc structures were observed among hybrid- and complex-type glycans, as well as sulfated *N*-glycans. Sulfated *N*-glycans are thought to potentially have a role in receptor binding and immune regulation (Wang et al. 2009). In this study by Zhao et al. (2020a), it was demonstrated that all 22 canonical *N*-glycosylation sites are utilized confirming the results by Watanabe et al. (2020b), with 19 of them having occupancies greater than 95%, and the 2 most C-terminal sites having reduced occupancy (N1173 of 52% and N1194 of 82%). Moreover, most high-mannose sites were reported to be dominated by the $Man_5GlcNAc_2$ structure, while sites N234 and N717 were dominated by the $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ structures, supporting the hypothesis by Casalino et al. (2020) that *N*-glycans at N165 and N234 modulate and stabilize the conformational dynamics of the S protein RBD responsible for ACE2 recognition.

O-glycans, which are involved in protein function and stability, have been observed on viral proteins and have been suggested to play roles in the biological activity of viral proteins (Andersen et al. 2020; Bagdonaite and Wandall 2018). Shajahan et al. observed two occupied *O*-glycosylation sites at the RBD of subunit S1 (expressed separately from the subunit S2) and this is the first report on the evidence for such glycan modification at a crucial binding location of the S protein (Shajahan et al. 2020). Zhao et al. confirmed *O*-glycans presence on SARS-CoV-2 expressed in trimeric form but with generally low occupancy of the sites (below 4% for most sites and 11% for T323) and detected 15 *O*-glycans released from the S trimer (Zhao et al. 2020a). Although it is unclear what is the function of these predicted *O*-linked glycans, the proposed theory is that they create a "mucin-like domain" that could shield the SARS-CoV-2 S protein epitope and allow immunoevasion.

Despite that the innate immune system has evolved strategies for responding to glycosylated pathogens, various mutations can alter the species specificity of the virus (Stevens et al. 2006), and the antigenicity of the surface proteins, as well as modulate its infectivity (Cotter et al. 2014). These mutations can impact the glycosylation degree of viral proteins by creating new glycosylation sites on the antigens (Altman et al. 2019). Varying surface antigen glycosylation is thus a mechanism by which new virus strains can evade the host immune response, making glycosylation elucidating an important step in targeting suitable antigens for vaccine development (Grant et al. 2020). Thus, it is obvious that glycosylation is relevant for the development of vaccines, and it is widely accepted that the lack of information about the glycosylation sites hampers the design of such vaccines (Wolfert and Boons 2013).

12.2.3 SARS-CoV-2 Cell Entry

As mentioned before, the glycan shield plays a critical role in hiding the S protein surface from molecular recognition. However, for SARS-CoV-2 to infect the host cell, the spike needs to recognize and bind to the ACE2 receptor (Fig. 12.1). The initial attachment of the CoV to the host cell occurs by binding of RBD in the viral S protein transiently sampling the functional conformation to ACE2 on the surface of target cells (Duan et al. 2020).

Both the viral spike and the hACE2 are glycoproteins and their glycosylation affects their interactions. The surface of the S protein is dominated by host-derived glycans, and each trimer displays 66 *N*-linked glycosylation sites (Watanabe et al. 2020b). Wrapp et al. show that SARS-CoV-2 has a 10–20 times higher affinity for the ACE2 receptor than the SARS-CoV-1, which may be related to glycosylation of the proteins (Wrapp et al. 2020). Interactions between the SARS-CoV-2 RBD and its ACE2 receptor have been revealed by several structures of

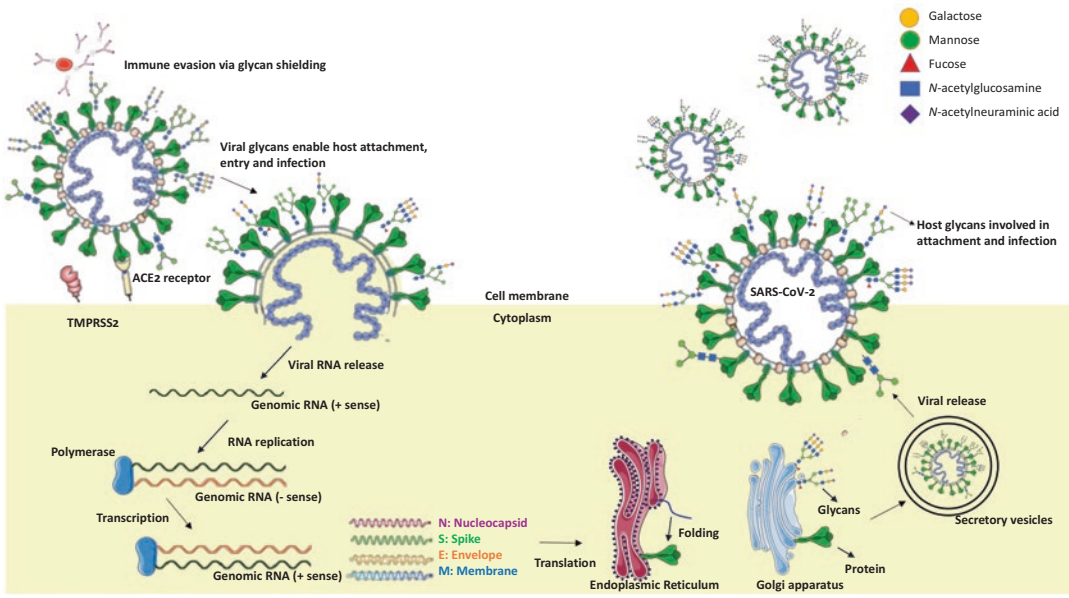

Fig. 12.1 SARS-CoV-2 cell entry and replication. Spike (S) glycoprotein is heavily glycosylated with high-mannose, hybrid, or complex-type *N*-glycans. Glycan shield enables immune evasion, as well as efficient interaction with host cell receptors, cell entry, and infection. Within the infected cells, SARS-CoV-2 S protein is post-translationally modified with host glycans before the virus is released in the plasma, potentially enabling higher infectivity of viral particles

ACE2 in complex with RBD (Shang et al. 2020; Lan et al. 2020; Wang et al. 2020a; Yan et al. 2020a). Structurally, RBD consists of a core and an external subdomain (Lan et al. 2020; Wang et al. 2020a). An extended loop, which lies on one edge of the core subdomain, contains almost all the amino acids of the SARS-CoV-2 RBD that interact with ACE2 and is referred to as the receptor-binding motif (RBM) (Lan et al. 2020).

Following the receptor binding, SARS-CoV-2 must access the host cell cytosol, which is generally accomplished by the TMPRSS2 protease (Hoffmann et al. 2020b), followed by fusion of the viral and cellular membranes. As mentioned before, the furin cleavage site between S1 and S2 subunit is believed to prime the spike for infection (Davidson et al. 2020; Belouzard et al. 2009). Viral entry through membrane fusion is initiated by envelope glycoproteins through molecular-recognition events involving cell surface receptors, which are often mediated by specific *N*-glycan epitopes (Watanabe et al. 2019; Wei et al. 2003; Go et al. 2015), resulting in the release of the viral genome into the cytoplasm. It has been proposed that inhibiting the interaction between RBD and ACE2 might be useful in treating SARS-CoV-2 infection.

Some studies reported the association of mannose-binding lectin (MBL) gene polymorphisms with susceptibility to SARS-CoV infection, although the role of MBL in SARS-CoV infection is not fully understood. MBL is a C-type lectin that plays a role in innate immunity by binding to carbohydrates on the surface of bacteria, viruses, fungi, and protozoa, where it activates the complement (Dommett et al. 2006; Van Asbeck et al. 2008). Zhou et al. observed inhibition of SARS-CoV by MBL and identified a single *N*-linked glycosylation site on SARS-CoV S protein that is critical for the specific interactions with MBL (Zhou et al. 2010). Keyaerts et al. showed that mannose-specific lectins can interfere with the S protein/ACE2 interaction and demonstrated its antiviral activity against SARS-CoV by blocking virus attachment to its receptor (Keyaerts et al. 2007). Recently, it was found by

Gao et al. (2020) that the SARS-CoV-2 N protein binds to mannose-binding protein-associated serine protease 2 (MASP-2), the key serine protease in the MBL pathway of complement activation, resulting in aberrant complement activation and aggravated inflammatory lung injury.

12.3 ACE2 Glycosylation

ACE2 is an 805-amino-acid type I integral membrane metalloproteinase anchored to the cell membrane via the C-terminus hydrophobic region, while its extracellular N-terminal region contains an active site. ACE2 is a carboxypeptidase that cleaves a single hydrophobic/basic residue from the C-terminus of its substrates. It efficiently hydrolyses the potent vasoconstrictor angiotensin II to angiotensin and with that participates in the renin–angiotensin system (Zhao et al. 2020a; Tikellis et al. 2011; Warner et al. 2004). Also, it serves as the receptor for SARS-CoVs attachment and fusion with host cells (Hoffmann et al. 2020a) and has been implicated in hypertension, cardiac function, and diabetes (Warner et al. 2004).

The hACE2 has six potential *N*-glycosylation sites at positions N53, N90, N103, N322, N432, and N546 resulting in a mass of 120 kDa, with glycans constituting around 35 kDa (Warner et al. 2004). Earlier studies of somatic ACE and testicular ACE suggested that glycan synthesis had a crucial role in ACE folding and intracellular targeting facilitating the expression, maturation, and activity of soluble and membrane ACE (Orth et al. 1998; Naim 1993; Gordon et al. 2003; Yu et al. 1997; Sadhukhan and Sen 1996). Moreover, it was shown that glycosylation site N53 had been conserved in rat ACE2, as well as in human, mouse and rabbit testicular ACE, and *Drosophila* Ance and Acer, which suggested that N53 site was essential for maintaining ACE2 activity (Warner et al. 2004). However, the functional significance of each glycosylation site for hACE2 processing, cell surface targeting, and activity has not been elucidated until recently.

Zhao et al. analyzed *N*-glycosylation of the soluble version of hACE2 expressed and purified from HEK293 cells and showed that site occupancy was high (>75%) at all six *N*-glycosylation sites. The majority of detected glycans were of complex type with limited high-mannose and hybrid-type glycans, and each site showed significant microheterogeneity (Zhao et al. 2020a). *O*-glycans were also detected at S155 and several S/T residues at the C-terminus of ACE2 but with low site occupancy levels at all sites (<2%) (Zhao et al. 2020a).

Glycosylation of ACE2 was shown to directly modulate SARS-CoV-2 and ACE2 interactions. Based on molecular dynamics simulations, ACE2 glycans at sites N90 and N322 are predicted to form interactions with S protein. Glycan–glycan interactions were observed in these simulations between ACE2 glycan at N546 and S protein glycans at N74 and N165. Of note, based on naturally occurring variants in the human population, *N*-glycosylation site N546 is predicted not to be present in 3 out of 10,000 humans (Zhao et al. 2020a).

Glycan–glycan interactions could alter the affinity of the SARS-CoV-2 and ACE2 interaction and modulate infectivity adding another layer of complexity for interpreting the impact of glycosylation on individual COVID-19 susceptibility. Hence, understanding the impact of glycosylation on ACE2 and its binding to S protein is important in the development of ACE2 as a potential therapeutic against a glycan-dependent epitope. Manipulation of ACE2 glycosylation could be a tool to create more potent and better competitive inhibitors of S protein binding.

12.4 Immunoglobulin Glycosylation

One of the key players in immunity and our first line of defense against foreign pathogens are Abs or Igs. There are five classes of Igs: IgG, IgM, IgA, IgE, and IgD, where IgG is the most abundant Ab in human plasma. All Igs are comprised of two 50–77 kDa class-specific heavy chains joined by one or more disulfide bonds and each one of them is connected via a disulfide bond to a 25 kDa light chain, creating a Y-shaped structure.

The antigen-recognition region (Fab fragment) of an Ig binds a specific antigen, while the Fc fragment interacts with Fc receptors (FcRs) on the surface of various immune cells facilitating immune response. While IgA, IgD, and IgG isotypes each have a flexible linker containing *N*- and *O*-glycans separating the Fab and Fc regions, IgM and IgE lack this hinge region and are thus more rigid in structure. IgA and IgG can be further divided into two (IgA1–2) and four (IgG1–4) subclasses, respectively. All four human IgG subclasses have two variable biantennary glycans attached at the conserved N297 site of the constant heavy 2 (CH2) domain on each of its heavy chains. The other Igs are more heavily glycosylated (de Haan et al. 2020).

Most of the IgG glycans are classified as *N*-glycans. The Fab region contains no conserved glycosylation sites, but it is estimated that about 15–20% of IgG molecules carry one or more *N*-glycosylation sites in the Fab variable regions (Zhu et al. 2002, 2003; Jefferis 2005; Dunn-Walters et al. 2000; Anumula 2012). Fab glycans are also suggested to play a role in the modulation of the immune response (van de Bovenkamp et al. 2016). Also, glycans can alter IgG's structural stability, conformation, and half-life, as well as its effector functions (Kao et al. 2015; Gornik et al. 2012; Dekkers et al. 2017; Bruggeman et al. 2017). Moreover, it is now generally accepted that the *N*-glycan composition of IgG Fc-domain is highly variable and has functional consequences (Jefferis 2009; Dekkers et al. 2018; Vidarsson et al. 2014). Indeed, complete removal of IgG Fc *N*-glycans results in the loss of both the pro- and anti-inflammatory activity of IgG (Albert et al. 2008).

Among all serum proteins, glycosylation of IgG is the most studied in different diseases (Gudelj et al. 2018). Due to the presence of two different glycans at the two CH2 domains of the same IgG molecule, Fab glycosylation, variable monosaccharide composition, and a different glycoprofile among the four IgG subclasses, the IgG glycome is known for its heterogeneity. As a result, several hundred differentially glycosylated IgG variants can be present in a person at any given time (Pučić et al. 2011; Seeling et al. 2017; Wuhrer et al. 2007). Under homeostatic conditions, IgG glycome composition shows little variation within an individual, but it can be quickly changed in cases of a disease (Gornik et al. 2009; Novokmet et al. 2014). IgG glycome composition in healthy people is influenced by age, gender, and hormones (Pučić et al. 2011; Baković et al. 2013; Ruhaak et al. 2010; Yamada et al. 1997; Engdahl et al. 2018; Jurić et al. 2020). Moreover, a high level of variation in the IgG *N*-glycome composition was found within human populations (Gornik et al. 2012; Pučić et al. 2011; Štambuk et al. 2020) and is affected by both the genetic and epigenetic factors (Lauc et al. 2013; Menni et al. 2013; Wahl et al. 2018a, b).

In healthy adults, IgG-G0 (agalactosylated structures) and IgG-G1 (monogalactosylated structures) each represent on average about 35% of total IgG Fc glycan structures while IgG-G2 (digalactosylated structures) makes up around 15% (Arnold et al. 2007; Huffman et al. 2014). The proportion of galactosylated structures is the most variable IgG glycosylation trait on the population level (Gornik et al. 2012; Huhn et al. 2009), but within an individual is relatively stable (Novokmet et al. 2014). IgG galactosylation can quickly change from an "on" to "off" mode in acute inflammation (Novokmet et al. 2014). An increased abundance of agalactosylated IgG glycans has been found in patients suffering from various diseases (Wahl et al. 2018a; Barrios et al. 2016; Gao et al. 2017; Vadrevu et al. 2018; Trbojević Akmačić et al. 2015).

There are observations that galactosylation of IgG acts as a modulator of its inflammatory activity (Banda et al. 2008). It was found that Fc galactosylation of IgG1 immune complexes is necessary for the efficient initiation of the anti-inflammatory signaling cascade through FcγRIIB binding (Karsten et al. 2012). Furthermore, it has been reported that highly galactosylated immune complexes inhibit the pro-inflammatory activity of the complement component C5a (Mihai and Nimmerjahn 2013). On the other hand, there are reports of IgG galactosylation acting in a pro-inflammatory manner, where terminal galactoses enhance IgG's affinity for activating FcγRs (Dekkers et al. 2017; Hodoniczky et al. 2005;

Peschke et al. 2017). These data, contradictory at first sight, indicate that classifying IgG galactosylation and IgG glycosylation, in general, should not be considered on its own, without considering the rest of the glycoprofile and biological context.

IgG-S (mono- and disialylated structures) represents about 10–15% of total IgG Fc glycan structures in healthy adults (Huffman et al. 2014). In contrast to galactosylation, terminal Sia appear to serve as a switch between IgG pro- and anti-inflammatory activity in cases of homeostasis disturbance, but the mechanisms through which they act are still not fully enlightened. Sia bound to an Fc glycan act through activating type I FcγRs, type II (lectin) receptors, and C1q complement component. Sialylated IgG has a decreased affinity for activating FcγRs expressed on the surface of innate immune cells, leading to a reduction in their activation and pro-inflammatory cytokine release (Seeling et al. 2017; Maverakis et al. 2014). Although pathways initiated by binding of sialylated IgG to lectin receptors are still not clear (Seeling et al. 2017; Karsten et al. 2012; Yabe et al. 2010; Kaneko et al. 2006), it is considered that binding of sialylated IgG to lectin receptors contributes to inflammation resolution by the release of T-helper 2 cytokines and the subsequent increase in the activation threshold of adaptive and innate immune cells (Hess et al. 2013). The effect of IgG sialylation on C1q binding, pro-inflammatory IgG effector functions, and the release of pro-inflammatory mediators remains controversial (Dekkers et al. 2017; Quast et al. 2015).

IgG contains a Fuc attached to the GlcNAc in the IgG glycan core structure (Baković et al. 2013; Zauner et al. 2013). The presence of a high proportion of core fucosylated IgG represents a "safety switch" in homeostasis (Scanlan et al. 2008) and IgG variants without core fucosylation cause antibody-dependent cellular cytotoxicity (ADCC), via increased IgG-Fc-receptor IIIa (FcγRIIIa) affinity (Ackerman et al. 2013; Wang et al. 2017).

About 10–15% of IgG glycans contain a bisecting GlcNAc (Arnold et al. 2007; van de Bovenkamp et al. 2019). At the level of glycan synthesis, the addition of core Fuc and bisecting GlcNAc are partially opposing processes (Benedetti et al. 2017). Thus, it is often difficult to interpret the individual functional roles of these two modifications in a particular disease. For example, higher levels of bisecting GlcNAc on IgG, similar to lower levels of core fucosylation, are often associated with a higher affinity for FcγRs, and, consequently, with enhanced ADCC and other immune cells effector functions (Arnold et al. 2007; Davies et al. 2001; Shinkawa et al. 2003).

12.4.1 Immunoglobulin Glycosylation in COVID-19 Infection

In addition to their function as a "glycan shield" facilitating host immune evasion, viral glycan epitopes can evoke specific Ab responses. Considering the key role of Igs in immunity, it is not surprising that IgG glycosylation has been studied in COVID-19 infection as one of the factors influencing COVID-19 severity.

Larsen et al. have explored Fc-glycosylation of total- and antigen-specific Abs in SARS-CoV-2 infection and observed significantly higher afucosylation of anti-S protein IgG in severe COVID-19 patients (Larsen et al. 2020). However, overall serum IgG Fc-fucosylation had usual high levels. This unique elevated level of afucosylated IgG glycoforms that have elevated FcγRIII activities has been previously observed in patients infected with HIV and Dengue virus (Ackerman et al. 2013; Wang et al. 2017), and in alloantibodies against red blood cells (RBCs) and platelets (Kapur et al. 2014a; b; Wuhrer et al. 2009). Interestingly, low IgG core fucosylation in Dengue infection has been associated with enhanced pathology due to excessive FcγRIIIa-activation, and anti-HIV Abs have been suggested to be a regulated feature of infection (Ackerman et al. 2013; Wang et al. 2017).

Different effector functions of Abs are modulated by Ab-intrinsic characteristics, such as subclass, allotype, isotype, and glycosylation (Vidarsson et al. 2014). In severely ill COVID-19

patients IgG afucosylation likely amplified the IgG effector function to promote pro-inflammatory cytokine production, of which COVID-19-associated cytokines such as IL-1β, IL-6, and TNF are most pronounced (Blanco-Melo et al. 2020; Del Valle et al. 2020). While decreased fucosylation increases the infection of cells by a process known as antibody-dependent enhancement (ADE) (Halstead 2015), there is little evidence for ADE infection in COVID-19 (Zohar and Alter 2020). Larsen et al. hypothesize that increased afucosylated IgG in COVID-19 patients most likely results from excessive immune activation. The combination of decreased fucosylation and increased galactosylation of IgG is known to particularly increase the affinity for FcγRII (Vidarsson et al. 2014). In addition to human alveolar macrophages, FcγRs are expressed by various myeloid immune cells (Hoepel et al. 2020), as well as airway epithelial cells (Golebski et al. 2019), which are one of the main target cells of infection by SARS-CoV-2 (Chua et al. 2020).

Petrović et al. showed differences in IgG glycome composition between patients with mild and severe COVID-19, where the level of bisecting GlcNAc was decreased in severe patients (Petrović et al. 2020). As mentioned before, higher levels of bisecting GlcNAc on IgG are often associated with increased FcγRIII binding and enhanced ADCC explaining a more pro-inflammatory effector function of IgGs (Irvine and Alter 2020). These findings were also observed in Larsen et al. study (2020), where anti-SARS-CoV-2 Abs have decreased bisecting GlcNAc. Interestingly, in the same study, anti-SARS-CoV-2 Abs had higher galactosylation and sialylation relative to the total IgG glycome composition, which is the opposite of what Petrović et al. observed implicating a potential pre-existing risk factor for more severe COVID-19. However, Chakraborty et al. found that IgG1 against the RBD of the SARS-CoV-2 S protein from COVID-19 patients had significantly lower core fucosylation, galactosylation, and bisecting GlcNAc compared to healthy individuals, suggesting that these differences may be individual (Chakraborty et al. 2020).

As mentioned before, glycans represent one of the main defenses against various pathogens and the repertoire of glycans changes with age and disease status of an individual (Krištić et al. 2014). Also, an earlier research study has shown that age and excess adiposity are risk factors for severe disease and mortality in people with COVID-19 (Seidu et al. 2020). Depending on the extent of galactosylation, sialylation, and fucosylation of its glycans, IgG will activate complement, activate ADCC, or even have an anti-inflammatory action (Gudelj et al. 2018). These findings indicate Ab glycosylation is a significant factor in inflammation and protection in enveloped virus infections including COVID-19.

12.5 ABO Blood Group

Glycans are one of the molecular bases of inter-individual differences within the human population and the existence of inter-individual differences serves us as one of the ways of defense against viruses. An example of inter-individual differences are ABO blood groups, in which diversity arises precisely from different glycans on the surface of blood cells.

Histo-blood group antigens are present on erythrocytes and epithelial cells (Marionneau et al. 2001). The antigenic determinants of A and B blood groups are trisaccharide moieties GalNAcα1,3-(Fucα1,2)-Galβ- and Galα1,3-(Fucα1,2)-Galβ-, respectively, while O blood group antigen is Fucα1,2-Galβ- (Zhao et al. 2020b). The connection of ABO blood group and viral infection susceptibility has been previously reported (Boren et al. 1993; Lindesmith et al. 2003; Batool et al. 2017), and it was shown that blood group O individuals were less likely to become infected both by SARS-CoV-1 (Cheng et al. 2005; Guillon et al. 2008; Greenwell 1997) and SARS-CoV-2 viruses (Zhao et al. 2020b; Zietz and Tatonetti 2020).

The ABO blood type trait reflects polymorphisms within the ABO gene. Cao et al. (2020) identified significant associations between genetic variants and immune phenotypes in SARS-CoV-1 infection that followed the 2003

outbreak (Zhao et al. 2011; Chan et al. 2007). Furthermore, red blood cell count, hemoglobin concentration, hematocrit (van Rooij et al. 2017; Kanai et al. 2018; Kamatani et al. 2010; Astle et al. 2016), von Willebrand factor (Sabater-Lleal et al. 2019; Van Loon et al. 2016; Williams et al. 2013), coronary artery disease (Nikpay et al. 2015; Nelson et al. 2017), ischemic stroke (Malik et al. 2018), type 2 diabetes (Xue et al. 2018; Scott et al. 2017; Mahajan et al. 2018), and venous thromboembolism (Klarin et al. 2017, 2019; Lindström et al. 2019) are conditions relevant for COVID-19.

Zhao et al. explored the ABO blood group distribution in a cohort of 2173 COVID-19 patients compared to healthy controls (Zhao et al. 2020b). They found blood group O had the lowest risk for the infection, while group A associated with a higher risk for acquiring COVID-19. Similarly, using a meta-analysis of data from Italy and Spain, Ellinghaus et al. found a higher risk of COVID-19 among A and a lower risk among O blood types (Severe Covid-19 GWAS Group 2020). Zietz et al. demonstrated an identical association (Zietz and Tatonetti 2020).

Anti-A or -B blood group Abs have long been suspected to play a role in antiviral immunity since viruses may carry ABO structures as terminal carbohydrate motifs of their envelope glycoproteins (Greenwell 1997). Arendrup et al. showed that monoclonal anti-A Ab neutralized HIV produced by lymphocytes, but this was the case only for anti-A Ab from blood group A donors (Arendrup et al. 1991). Also, Neil et al. showed that anti-A or -B Abs from human serum were interacting with HIV vectors containing ABO histo-blood group sugars and facilitating its complement-mediated inactivation analogous to ABO incompatibility (Neil et al. 2005). According to these data, it is expected that during an outbreak, blood group O individuals had a lower risk of infection than non-blood group O individuals, which was observed during the outbreak of SARS in Hong Kong (Cheng et al. 2007). To prove the hypothesis of the protective role of natural anti-A or -B Abs from blood group O individuals in blocking SARS-CoV entry into target cells, Guillon et al. demonstrated that anti-A or -B Abs from blood group O, B, and A individuals could bind to the S protein and block its interaction with ACE2 (Guillon et al. 2008). Given the receptor ACE-binding similarity between SARS-CoV and SARS-CoV-2 (Hoffmann et al. 2020b; Wan et al. 2020a), the lower susceptibility of blood group O and higher susceptibility of blood group A individuals for COVID-19 could be linked to the presence of natural anti-blood group Abs, particularly anti-A Ab (Zhao et al. 2020b).

Glycosylation of viral glycoproteins requires glycosylation machinery of an infected cell. Viral particles in cells expressing histo-blood group antigens can be labeled with these antigenic motifs, which may have a protective role (Seymour et al. 2004). There may also be other mechanisms in addition to ABO blood group-differentiated susceptibility for COVID-19 that require further studies. As mentioned before, age and environmental factors are among the risk factors for susceptibility and severity of COVID-19 disease. Several studies showed that an unhealthy lifestyle, obesity, and smoking can increase mortality, indicating that biological age is more relevant than chronological age in predicting COVID-19 disease severity (Lauc and Sinclair 2020). Also, several studies demonstrate the effect of temperature and humidity on respiratory virus stability and transmission rates (Kifer et al. 2021; Audi et al. 2020).

12.6 SARS-CoV-2 Vaccines and Therapy

Due to the rapid global spread of SARS-CoV-2 and the high mortality rate, the development of vaccines and therapeutics is urgently needed. Research studies that followed the SARS-CoV-1 and MERS-CoV epidemics have laid a foundation for current efforts in the COVID-19 pandemic, including the importance of the S and ACE2 glycoprotein in vaccine development. A critical step between the virus and the host cell is the binding of S glycoprotein to the ACE2 receptor on the surface of human cells. As mentioned above, both the S glycoprotein and ACE2 recep-

tor are extensively glycosylated (Zhao et al. 2020a), and site-specific *N*-linked glycosylation of SARS-CoV-2 S glycoprotein has indicated that each of these glycosylation sites can be occupied by different glycans, which extends epitope diversity (Walls et al. 2020; Watanabe et al. 2020b; Shajahan et al. 2020; Zhao et al. 2020a).

Although glycans determine the function of many glycoproteins, as well as S and ACE2 glycoproteins, they are often overlooked. Some of the reasons are their chemical complexity and limited throughput and sensitivity of existing analytical instruments. Also, glycans determine a significant part of the overall structure, especially in the field of host/pathogen interactions, where glycan diversity is used by both pathogens to escape the immune system response and host to evade recognition by pathogens. Furthermore, glycans have significant conformational flexibility, which contributes to the overall conformational dynamics of the molecule that can generate potential drug-binding sites.

There are a dozen potential drugs to treat SARS-CoV-2 infection already in clinical trials, such as chloroquine (CQ), and its alternative hydroxychloroquine (HCQ), as well as neutralizing Abs. Also, antivirals, a class of small molecules that function as inhibitors of a virus life cycle, are drug candidates against SARS-CoV-2. Because of similarities between replication mechanisms of different viruses, some antivirals can be repurposed against various viral infections. Many clinical trials using experimental antiviral drugs are currently underway. A small proportion of them is aimed at repurposing existing antivirals, including arbidol (umifenovir) (Chen et al. 2020a), a lopinavir/ritonavir (Yan et al. 2020b), favipiravir (Chen et al. 2020a), and remdesivir (Hayden and Shindo 2019).

12.6.1 The S Protein as a Vaccine Target

Since the SARS-CoV-2 genome sequence was released (Wu et al. 2020), SARS-CoV-2 vaccine candidates based on various vaccine platforms have been developed (Pillaiyar et al. 2020; Le et al. 2020). Most of these vaccine strategies are based on the full-length S glycoprotein. Thus, understanding glycosylation of SARS-CoV-2 S protein, its binding to the receptor, and subsequent SARS-CoV-2 membrane fusion, cell entry, and replication are crucial (Watanabe et al. 2020b; Pillaiyar et al. 2020).

Although the SARS-CoV-2 S protein shares ~76% of amino acid sequence identity with SARS-CoV-1, the RBM is less conserved (Jaimes et al. 2020). It is assumed that the RBM has the most immunodominant neutralizing epitope of the whole S protein, which can cause strong neutralizing Ab responses. Vaccine candidates based on the full-length S protein could cause neutralizing Ab responses against COVID-19, but they may also induce harmful immune responses, including ADE of SARS-CoV-2 infection (Karthik et al. 2020; Arvin et al. 2020). Another concern for vaccine candidates based on the full-length S glycoprotein of SARS-CoV-2 is that the S1 subunit could spontaneously dissociate from the S glycoprotein, resulting in ineffective Ab responses, as documented for HIV-1 (Duan et al. 2015). Also, the virus can suppress the immune response by hijacking the host cellular machinery for replication, form a glycan shield that is composed of familiar host glycans (Grant et al. 2020), and mask the immunogenic epitopes targeted by neutralizing Abs (Watanabe et al. 2020a, b).

In addition to the spike head and RBD (Yuan et al. 2020; Jiang et al. 2020), the stalk is another possible target for Abs. However, Casalino et al. showed that the spike head is overall less shielded by the glycans than the stalk, even though 19 *N*-glycans essentially camouflage the spike head region, but only 3 *N*-glycosylation sites (N1158, N1174, and N1194) shield the stalk (Casalino et al. 2020). The stalk surface is well protected by sialylated and fucosylated tetrantennary glycans, which are found to be very effective in shielding large molecules making the stalk a potentially more challenging therapeutic target than the spike head (Casalino et al. 2020). Furthermore, other studies showed that glycans at N1174 and N1198 are tetrantennary fucosylated structures, while the N1198 glycosylation

site has sialylated glycan structures (Watanabe et al. 2020a, b).

A growing number of nAbs targeting the SARS-CoV-2 S glycoprotein with high potency has been isolated from convalescent plasma (CP) (Schmidt et al. 2020) as well as through animal vaccination (Hansen et al. 2020). It is widely accepted that the S protein of SARS-CoV-2 is the most promising immunogen for inducing protective immunity (Zhang et al. 2020a). To obtain these nAbs, RBD-specific memory B cells were sorted and IgG heavy and light variable regions cloned to express recombinant forms of the corresponding Abs (Chen et al. 2020b; Ju et al. 2020). Chen et al. demonstrated that nearly all nAbs derived from serum of recovered patients bound to S1 and RBD, but only three inhibited ACE2/RBD binding (Chen et al. 2020b). Since SARS-CoV-1 and SARS-CoV-2 consensus sequences share ~76% identity (Tai et al. 2020), a wide range of SARS-CoV-1 nAbs has been tested for cross-reactivity with SARS-CoV-2 (Pinto et al. 2020).

Hyperinflammatory responses and higher levels of inflammatory cytokines, including IL-6, IL-8, and IL-10, have been shown to correlate with COVID-19 severity (Feng et al. 2020; Diao et al. 2020; Gong et al. 2020; Moore and June 2020; Wan et al. 2020b; Xu et al. 2020). Furthermore, it has been shown that patients with severe forms of SARS-CoV-2 have neutralizing IgG autoantibodies against type I interferons (IFNs) (Bastard et al. 2020). The drivers of the cytokine storm remain to be established, but the heterogeneous response between patients suggests that the SARS-CoV-2 receptor, hACE2, is involved (Hirano and Murakami 2020). The IL-6 has been identified as a critical cytokine in COVID-19 in preliminary research studies, and, consequently, monoclonal Abs that target the IL-6 signaling pathway have been proposed as therapeutic candidates (Moore and June 2020). The commercial anti-IL-6R Ab sarilumab (Kevzara), tocilizumab (Actemra), and the anti-IL-6 Ab siltuximab (Sylvant) are now being tested in ongoing clinical trials for efficacy in managing COVID-19 cytokine storm.

12.6.2 ACE2 as a Therapeutic Target

Recombinant Fc fusion proteins can improve in vivo efficacy, gain immunoreactive functions, and have been widely used in modern biopharmaceuticals (Shapiro 2013; Collins et al. 2016). On the other hand, ACE2, a key player in CoV infection, plays a central role in the homeostatic control of cardiorenal actions. Since this enzyme was identified as the SARS-CoV-2 receptor (Hoffmann et al. 2020a), several approaches to address ACE2-mediated infection have been described (Zhang et al. 2020b; Li et al. 2020), to prevent host cell entry and viral replication.

Soluble recombinant ACE2 (rACE) (Monteil et al. 2020) and ACE2 with an Fc fragment (ACE2-Fc) of the human IgG1 (Lei et al. 2020; Iwanaga et al. 2020) have been shown to have potential applications in the treatment of SARS-CoV-2 infection in vitro. The neutralizing effect of ACE2 remained when two active-site H residues of ACE2 were modified to N residues (Lei et al. 2020). Still, the authors caution that this model might not be as efficient for the live SARS-CoV-2 neutralization. One safety concern of ACE2 fusion proteins is that they may have systemic cardiovascular side effects.

The use of soluble extracellular domains of ACE2 as competitive inhibitors for SARS-CoV-2 infection emphasizes the critical need for understanding the glycosylation profile of ACE2 so that optimal therapeutics can be produced (Monteil et al. 2020; Lei et al. 2020).

Due to the roles of glycosylation in mediating SARS-CoV-2 and ACE2 receptor binding and antigenic shielding, it is important to monitor the evolution of the virus to determine if existing glycosylation sites are lost or new sites have emerged with selective pressure that might alter the efficacy of vaccines.

12.6.3 SARS-CoV-2 Glycosylation Targeting

In the cell, SARS-CoV-2 S protein is posttranslationally modified with high-mannose, hybrid, and complex glycans, with some *N*-glycosylation

sites predominantly being occupied with high-mannose glycans. Recently, it was suggested that interfering with SARS-CoV-2 glycosylation to reduce the S protein glycan shield would result in antigenic peptide sequences being more accessible to Abs (Bliard 2020). Higher exposure of viral antigenic sites for host circulating Abs could potentially boost the immune system and fight COVID-19.

Man derivatives as deoxy-D-Man and fluoro- or chloro-substituted analogs could potentially be recognized and bound by oligomannose synthases during dolichol-phosphate glycan synthesis and incorporated into the growing glycan. These Man analogs would introduce deficiency of –OH groups at key branching or elongating positions and prevent the further growth of oligomannose-terminal glycan. Resulting truncated glycans would leave larger S protein surface unshielded and vulnerable to host Abs recognition (Bliard 2020).

However, this kind of intervention would also interfere with the normal cell glycosylation process, and despite promising potential for disrupting SARS-CoV-2 glycan maturation during replication in infected cells, it should be studied with extreme caution.

12.6.4 Therapeutic Immunomodulation for COVID-19

Drug CQ and its derivative HCQ, a well-known antimalarial drug, are also possible therapeutics for COVID-19. As well as previously described drugs, their application in COVID-19 therapy originates from their past use as antivirals (Savarino et al. 2003), including SARS-CoV-1 infection (Vincent et al. 2005; Keyaerts et al. 2004). CQ and HCQ interfere with lysosomal activity and have been reported to have immunomodulatory effects (Yao et al. 2020; Wang et al. 2020b). Furthermore, studies involving in vitro infection of host cells with SARS-CoV-2 demonstrated that both CQ and HCQ significantly impact endosomal maturation, resulting in increased sequestration of virion particles within endolysosomes. Both compounds inhibit SARS-CoV-2 infection in vitro increasing the pH of intracellular organelles and altering the glycosylation profile of ACE2 receptors and S protein, as well as interfering with virus replication (Liu et al. 2020; Zhou et al. 2020c).

It is suggested that a major mechanism for CQ antiviral effects is precisely glycosylation inhibition by specific interactions of CQ and sugar-modifying enzymes or glycosyltransferases. It was shown that CQ inhibits quinone reductase 2, and suggested by computer simulations that it can bind to the active site of its structural neighbor, UDP-*N*-acetylglucosamine 2-epimerase, which catalyzes the rate-determining step in the Sia biosynthesis pathway (Savarino et al. 2004, 2006). Similarly, HCQ was predicted to interfere with glycosylation of several proteins involved in the humoral immune response, probably also through inhibition of UDP-*N*-acetylglucosamine 2-epimerase (Brufsky 2020). Despite the widespread use of HCQ and CQ to treat COVID-19, few controlled clinical trials have been performed so far and thus the potential benefits of these drugs for COVID-19 remain controversial (Wang et al. 2020b; Liu et al. 2020; Zhou et al. 2020c; Gautret et al. 2020a, b; Clementi et al. 2020; Lover 2020; Hulme et al. 2020; Chen et al. 2020c).

12.6.5 Challenges

The development of a vaccine to protect against COVID-19 has progressed at an unmatched rate due to prior knowledge gained after SARS-CoV-1 and MERS-CoV outbreaks. However, many challenges lie ahead. The fact that COVID-19 is disproportionately more severe in older adults poses a question of vaccine efficacy in older individuals (Nikolich-Žugich 2018), as well as the possibility of ADE of COVID-19 (Cao 2020; Huang et al. 2020).

All these above mechanisms show the importance of understanding the role of glycosylation in the development of potential new treatments or new vaccines. Moreover, different vaccines induce different glycosylation patterns of Abs,

which affect their ability to activate effector functions and provide protection against future infections (Mehta and Alter 2017).

12.7 Perspectives

The appearance of SARS-CoV-2 in late 2019 became one of the major health problems in the world. SARS-CoV-2 survival and virulence are shown to be impacted by glycans, making glycobiology an area of interest in SARS-CoV-2 biology and COVID-19 infection. Due to their chemical complexity and limited sensitivity of analytical instruments, glycans have often been disregarded in research studies. Today glycobiology field has advanced in terms of available analytical technologies that facilitated understanding of the functional roles of glycans in various biological systems.

Both glycosylation of SARS-CoV-2 and Igs as key molecules in immunity have been studied in COVID-19 infection. However, inconsistencies in obtained results emphasize the impact of a context (e.g., expression system, protein form) on the obtained glycosylation profile of S glycoprotein. Ideally, SARS-CoV-2 glycosylation should be studied on virions harvested from airway cells or COVID-19 patients, as well as monitored in new emerging strains.

Equivalently, initial studies on Ig(G) glycosylation in COVID-19 patients have provided valuable knowledge on glycosylation changes and suggested potential mechanisms of Ig action in COVID-19 infection. Still, patient samples for these cohorts were mostly collected during the first wave of pandemics and mostly lack detailed patient characterization and follow-up, as well as matching healthy controls. Further longitudinal and more extensive studies including detailed patient clinical data are required to assess the importance of Ig glycosylation changes in COVID-19 predisposition, severity, and treatment.

Many viruses, such as HIV and influenza viruses, make use of glycans as entry receptors (e.g., Sia, histo-blood group antigens). The initial attachment of the SARS-CoV-2 to the host cell occurs by the interaction of viral S protein and ACE2 receptor on the surface of target cells. Both the viral S protein and the ACE2 are glycoproteins and with glycan–protein and glycan–glycan interactions affecting their binding. Analyzing site-specific glycosylation of both the S glycoprotein and ACE2 receptor, indicated that each glycosylation site can be occupied by different glycans, which extends epitope diversity. The impact of different S protein and ACE2 glycoforms on SARS-CoV-2 infectivity remains an open question.

The development of vaccines and therapeutics against SARS-CoV-2 is based on a foundation from previous SARS-CoV-1 and MERS-CoV epidemics. CQ, neutralizing Abs, and antivirals are among a dozen potential drugs to treat SARS-CoV-2. However, strong conclusions on their efficacy based on currently conducted clinical studies are still underway. Glycoprotein S and ACE2 receptor are also therapeutic targets. Detailed understanding of the impact of ACE2 glycosylation to SARS-CoV-2 S glycoprotein binding is an important next step for developing efficient therapeutics targeting this interaction. Moreover, it is important to monitor the SARS-CoV-2 evolution to determine if existing glycosylation sites are lost or new sites have emerged with selective pressure that might alter the virus infectivity and efficacy of vaccines.

These data suggest that detailed glycan analysis of the SARS-CoV-2 proteins, as well as ACE2 receptor, is very important for the development of a glycoprotein-based vaccine. Also, understanding of complex sialylated *N*-glycans and sialylated mucin-type *O*-glycans is useful for understanding the pathology of virus binding and the infection itself in future therapeutic possibilities.

Compliance with ethical standards.

Ethical Approval This work does not contain any studies with human participants or animals performed by any of the authors.

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The Role of Glycosylation in Inflammatory Diseases

13

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Abstract

The diversity of glycan presentation in a cell, tissue and organism is enormous, which reflects the huge amount of important biological information encoded by the glycome which has not been fully understood. A compelling body of evidence has been highlighting the fundamental role of glycans in immunity, such as in development, and in major inflammatory processes such as inflammatory bowel disease, systemic lupus erythematosus and other autoimmune disorders. Glycans play an instrumental role in the immune response, integrating the canonical circuits that regulate innate and adaptive immune responses. The relevance of glycosylation in immunity is demonstrated by the role of glycans as important danger-associated molecular patterns and pathogen-associated molecular patterns associated with the discrimination between self and non-self; also as important regulators of the threshold of T cell activation, modulating receptors signalling and the activity of both T and other immune cells. In addition, glycans are important determinants that regulate the dynamic crosstalk between the microbiome and immune response. In this chapter, the essential role of glycans in the immunopathogenesis of inflammatory disorders will be presented and its potential clinical applications (diagnosis, prognosis and therapeutics) will be highlighted.

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Inflammatory diseases · Glycosylation · T-cells · IBD · Glomerulonephritis · Myositis

13.1 Role of Glycans in Adaptive Immune Development

Protein glycosylation has the potential to take part in the majority of cellular processes and to regulate cell-fate decisions, activity, function, for instance. Adaptive immune cells play a central role in the orchestration of an inflammatory response, as well as in its resolution. One of the factors contributing to inflammation is the recognition of non-self or altered biological material. T and B cells acquire the potential to recognize such signals during their early stages of development and glycans have been shown to play an essential role in these processes. The next section discusses the work on the influence of glycans in developmental checkpoints.

13.1.1 Glycans in Early T Cell Development

T cell development is one of the major physiological processes that occur in complex organisms, which ensures the formation of a proper repertoire of T cell receptors (TCRs), fundamental in immune responses (Koch and Radtke 2011). T lymphocytes develop in the thymus from a multistep program characterized by the sequential rearrangement of the *Tcrb* and *Tcra* loci in combination with lineage and control steps (Takaba and Takayanagi 2017). As the several events of development are dependent on both cell-intrinsic and -extrinsic factors, the study of glycans constitutes a major developmental feature of knowledge (Marth and Grewal 2008; Pereira et al. 2018a, b).

The initial step of T cell development occurs in the bone marrow, where lymphoid progenitors exit to the bloodstream, then to enter into the corticomedullar tissue of the thymus, in which they start to expand and develop. The trafficking of thymus seeding progenitors (TSPs) requires the expression of P-selectin glycoprotein ligand-1 (PSGL-1) by those progenitor cells and its partner, P-selectin, by the thymic epithelium (Rossi et al. 2005). The glycosylation profile of PSGL-1, namely its α1,3 fucosylation, was shown to be required for its binding to P-selectin, and its absence, by the genetic deletion of the Fut4 and Fut7 fucosyltransferases, led to the impairment of TSPs homing into the thymus (Sultana et al. 2012). In the sialyltransferase *St8Sia-IV*$^{-/-}$ mouse model, a deficient thymic seeding was also observed, caused by inefficient progenitor bone marrow egression (Drake et al. 2009). Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$double negative 1 (DN1) population, which can give rise to multiple lymphoid lineages (Takaba and Takayanagi 2017). The major determinant responsible for the commitment of DN1 thymocytes to the T cell lineage is the presence of Notch signalling (Shah and Zúñiga-Pflücker 2014). The glycoprofile of Notch receptors (and ligands) was shown to regulate Notch-dependent intracellular signal transduction. These proteins are modified by the lunatic, manic and radical Fringe glycosyltransferases, which transfer *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans, in the repeats of the consensus epidermal growth factor-like (EGF-like) sequences present in the extracellular domain of Notch (Rampal et al. 2005). The presence of these glycans regulates the trafficking of the Notch receptors (Takeuchi et al. 2017) and its differential affinity for Notch-ligands (Rampal et al. 2005). It was shown that the mouse model of triple fringe deficiency had reduced the binding of Notch to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T cell subsets in the thymus (Song et al. 2016). The decisive commitment to the T cell lineage occurs at the DN3 stage, where a recombination-activating genes (RAG)-mediated productive rearrangement of the *Tcrb* leads to the expression of the ß chain of the TCR (TCRß) and the formation of a pre-TCR signalling complex (Takaba and Takayanagi 2017). Together with Notch and

IL-7, the pre-TCR signalling initiates ß-selection, by inducing the transition to DN4 cells. These cells then begin a round of multiple divisions, giving rise to the most represented thymocyte population, the $CD4^+CD8^+$double-positive (DP) cells.

The major function of the thymus as an organ is to generate an environment where randomly generated TCRs are probed for their reactivity and selected according to their self-reactive potential (Miller 2020). These biological processes occur in the DP stage, after the Tcra locus suffers rearrangements by the RAG complex, leading to the expression of a mature TCR (Shih et al. 2011). The new mature TCRs are then screened by thymic epithelial cells (TECs) by the specificity and binding strength for the presented MHC ligands. The initial process is named positive selection, where the DP population is enriched for cells that express an immunocompetent TCR. Afterwards, cells that display high levels of activation, which indicates self-reactive potential, are targeted for apoptosis, a process called negative selection. Finally, cells that go through both selections develop into $CD4^+CD8^-$ or $CD4^-CD8^+$single positive (SP) cells. One of the major contributions of glycans is represented by the ones of the CD4 and CD8 co-receptors. It was shown that chemical desialylation of CD8 mature cells increases CD8/MHC-I interactions (Daniels et al. 2001), and in fact, *ST3Gal1*$^{-/-}$ mice show a significantly altered TCR repertoire, indicating a role for sialylation in thymocyte selection (Moody et al. 2001). Furthermore, it was demonstrated that branching *N*-glycosylation expands the range of TCR signalling of positive selection by differentially controlling both the lower and upper limits of positively selected TCR–MHC–antigen interactions. This was pointed out to be due to decreased surface expression of CD4 and CD8 receptors, in *Mgat1* and *Mgat2* DP-conditional knockout models (Zhou et al. 2014).

Surface glycans also modulate galectin (gal-) binding, which in turn influence cellular functions (Rabinovich and Toscano 2009). Histological analysis of thymus from mice revealed differential galectin spatial distribution, suggesting functions in distinct developmental stages (Nio-Kobayashi 2018). Perillo et al. showed that gal-1 was able to induce apoptosis of human thymocytes in vitro, with high effect in combination with anti-CD3 stimulation, suggesting a role in negative selection (Perillo et al. 1997). Later on, Galvan et al. demonstrated that the downregulation of galectin-binding glycans occurs in positively selected DP thymocytes, which are resistant to gal-1-induced apoptosis (Galvan et al. 2000). Gal-3 was shown to regulate thymocyte-epithelial cell interaction, influencing developmental transitions. In fact, *Lgals3*$^{-/-}$ mice show decreased absolute numbers of thymocytes, with lower levels of proliferation (Oliveira-de-Abreu et al. 2018).

13.1.2 Glycans in Early B Cell Development

B cell lymphocytes are key players in the adaptive immune response, being essential regulators of immunity through the secretion of antibodies, soluble proteins with antigen specificity. B cell development is a highly regulated process that takes place in the bone marrow and the spleen (Hardy and Hayakawa 2001). Much like T cells, B cells undergo somatic gene rearrangement in the immunoglobulin loci, and are selected to ensure a self-tolerant repertoire of antibodies. Its development is crucial to ensure proper immune function throughout the life of the organism.

B cells are generated from common lymphocyte progenitors (CLPs) that remained in the bone marrow. As in DN1 thymocytes T cell lineage commitment, the CLP commitment to the B cell lineage is influenced by Notch signalling. CLPs require absent Notch signalling to develop into progenitor B cells (pro-B). Unlike the role of this pathway in T cell development (promoting effect), Notch has to be absent for B cell development to occur (Stanley and Guidos 2009). Glycosylation profiles of the Notch receptor and ligands regulate affinity and surface expression. Interestingly, conditional knockout of Lfng in thymocytes led to B cell development in the thymus (Koch et al. 2001).

When pro-B cells successfully rearrange the Igh locus, mediated by the RAG complex, they develop into precursor B cells (pre-B). The cells in this developmental stage express a pre-BCR complex, with no antigen specificity, which triggers a signalling cascade, driving proliferation. The pre-BCR receptor is composed by the rearranged immunoglobulin heavy chain that assembles with a surrogate chain, to ensure surface expression (Burrows et al. 2002). It was seen that the *N*-glycans of the rearranged immunoglobulin heavy chain influence this assembly and are specifically required for pre-BCR function (Übelhart et al. 2010). Moreover, core fucosylation was also demonstrated to play a role in pre-BCR formation (Li et al. 2012). Stromal gal-1 was shown to be a ligand of the pre-BCR, triggering its signalling pathway and enabling further B cell development (Gauthier et al. 2002). In fact, B cells in this stage are found in the stromal niches where gal-1 is enriched (Mourcin et al. 2011) and their development is compromised in the absence of gal-1(Espeli et al. 2009). The signalling activation of the pre-BCR cascade leads to the rearrangement of the immunoglobulin light chain, which results in the expression of a mature BCR, and development into the immature B cell stage.

Immature B cells are then screened for their autoreactive potential in the bone marrow. Cells reacting with low or high affinity suffer receptor editing, with a secondary rearrangement of their immunoglobulin light chain allele (Schatz and Ji 2011). Afterwards, BCR with affinity for self-peptides are negatively selected, and cells that express a tolerant BCR are positively selected, by which central B-cell tolerance is achieved (Nemazee 2017). Recently, it was shown that branched *N*-glycans are required for B cell selection (Mortales et al. 2020). By the conditional knockout of *Mgat1* in the B cell lineage, it was observed that branched *N*-glycan deficiency decreased the surface expression of the BCR co-receptor CD19, which inhibited positive selection. Moreover, the nerve growth factor IB (Nur77) was shown to be upregulated in immature B cells in the absence of branched *N*-glycans, indicating a role of threshold establishment, similarly to T cells (Mortales et al. 2020).

B cells that go through central selection migrate to the spleen and commit to either the marginal zone (MZ) or follicular cell fates, according to the strength of their BCR signal (Pillai and Cariappa 2009). Interestingly, mice deficient for CD22, a B cell siglec that binds α2,6-sialic acids, which inhibits BCR signalling, show decreased cellularity on the MZ B cell compartment (Samardzic et al. 2002). B cell homing was shown to be compromised in a *Cosmc*$^{-/-}$ genetic background, demonstrating the role of elongated *O*-glycans in this process (Zeng et al. 2020).

When resting B cells encounter an antigen, one of the major features of the humoral response takes place: the antibody diversification and maturation. This process occurs in germinal centres (GC) where the selection of B cells according to their antigen affinity. Cell clones which display high antigenic affinity, proliferate and differentiate into antibody-secreting plasma cells and memory B cells (Mesin et al. 2016). Galectins have been implicated in the regulation of BCR signalling, influencing therefore B cell germinal centre selection. Interestingly, loss of gal-3 resulted in increased B cell activation and spontaneous GC formation (Beccaria et al. 2018). It was also shown that both gal-1 and gal-8 promote plasma cell differentiation (Tsai et al. 2011). In fact, it was demonstrated that binding of gal-8 endorses antigen recognition by B cells, promoting the formation of the immunological synapse (Obino et al. 2018).

13.2 Role of Glycans in the Inflammatory Process

13.2.1 Glycans in Innate Immune Responses

Innate immune cells are the first line of defence against pathogens and play a major role in the inflammatory response. This immune cellular group comprises mast cells, phagocytes (dendritic cells (DCs), macrophages and neutrophils), NK cells and innate lymphoid cells (ILC). Their glycosylation profile should be taken into consid-

eration to understand their functions in an inflammatory environment or during an immune response. Trafficking and recruitment of innate cells to the sites of tissue injury are controlled through glycosylation-mediated recognition between leukocytes and endothelial cells. Moreover, the migration of these cells to the inflammation sites allows the accumulation of specific cytokines and chemokine cocktails which are also responsible for altering the glycosylation of surrounding cells. Moreover, this interplay between innate immune cells and the inflammatory environment is also mediated by specific glycan-recognizing receptors. It is clear that protein glycosylation plays a fundamental role in each major step of the inflammatory process—initiation, propagation and abrogation of inflammation—and the next section details the contribution of glycans in each one.

13.2.1.1 Glycans in Immune Cell Trafficking and Recruitment

Upon infection or tissue damage, endothelial cells exhibit cell surface changes in order to promote the migration and extravasation of immune cells to the site of injury. Roll, arrest and adherence steps are controlled through the interaction between endothelial selectins (E-Selectin and P-Selectin) and ligands present in leukocytes (Zarbock et al. 2011). Selectins are C-type lectins (calcium-dependent glycan-binding proteins) characterized by their ability to recognize and bind specific carbohydrates structures, such as Sialyl Lewis X (sLeX) (Schnaar 2016). Changes in cellular glycosylation affect selectin-binding, such as PSGL-1 (P-Selectin) or ESL-1 (E-Selectin), modulating the process of recruitment and homing of immune cells (Sperandio 2006). In fact, it was described that mice lacking *ST6Gal1* gene, which codes the sialyltransferase responsible for the addition of terminal α2,6-sialic acids, showed an impaired migration of immune cells toward draining lymph nodes (Zarbock et al. 2011). The hyaluronic acid receptor CD44 is also an important leukocyte ligand for endothelial E-Selection. CD44-E-Selectin interaction is highly dependent on the surface sialylation and fucosylation of its *N*-glycans (Kansas 1996). Thus, the activity of glycosyltransferases during this process of recruitment is vital to mount a correct in situ inflammatory response.

13.2.1.2 Glycans as Recognition Moieties (PAMPs and DAMPs)

After cellular migration to the injury site, the recognition of pathogens and/or injured host cells is an important step in the inflammatory process. Innate immune cells express a variety of cell surface receptors with the specific capacity of recognize 'danger' structures, known as pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP). PAMPs and DAMPs are often glycosylated biomolecules present in the surface of the pathogens or damaged cells, or even released to the extracellular space (Ablasser and Chen 2019). One of the mechanisms by which these molecules are recognized by innate immune cells is through carbohydrate-recognizing receptors, such as C-type lectins (CTLs). Antigen-presenting cells, such as macrophages and DCs, bear a diverse and robust collection of several C-type lectins that enable them to recognize several danger glycosignals (Brown et al. 2018). The Dectin-1 CTL recognizes β-linked glucose polymers, a major constituent of pathogen cell walls, and is able to trigger cellular activation with its intracellular domain, through the NF-κB signalling cascade (Ferwerda et al. 2009). Dectin-2 recognizes mannose residues displayed by pathogens and host cells and is able to interact with FcγRs through its extracellular domain, inducing cellular activation (Hollmig et al. 2009). Interestingly, *Dectin-2*$^{-/-}$mice have shown a decreased susceptibility to house dust mite-induced lung inflammation, highlighting the importance of this first mechanism of defence (Parsons et al. 2014). Another well-known CTL, mostly studied in the context of cancer, is dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN). This CTL is mainly expressed in immature DCs, monocytes and macrophages, and recognizes high-mannose and fucose residues. DC-SIGN assists also in the

antigen recognition leading to the maturation of DCs (RodrÍguez et al. 2018;Brown et al. 2018).

13.2.1.3 Glycans in Innate Immune Cell Function

During an inflammatory process, APCs and monocytes migrate to the tissue where they encounter the inflammatory agent (tumour cell, pathogen, for instance). After recognition, in which specific receptors bind to the surface molecules of the agent, DCs suffer a shift on its phenotype, characterized by increased expression of MHC-II, costimulatory molecules (CD80, CD86) upregulation, as well as increased cytokine and chemokine secretion. This protein shift is accompanied with changes in surface glycosylation that distinguish mature (mDCs) and immature (iDCs) DCs. Maturation of DCs is associated with an increase of elongated poly-*N*-acetyllactosamine chains with terminal α2,3-sialic acid and fucose (Bax et al. 2007). These glycan expression alterations represent an important mechanism that regulates DC functions, such as antigen presentation to T and B cells in the lymph nodes. Higher abundance of terminal sialic acid was shown to enable the *trans* binding of sialoadhesins (CD22), expressed by B cells, supporting a potential DC-B cell interaction during antigen presentation (Varki and Gagneux 2012). Moreover, the increased presence of elongated poly-*N*-acetyllactosamine chains favours galectins binding, which in turn have been shown to regulate cell adhesion, cell activation, chemoattraction, cell growth and apoptosis in DCs (Videira et al. 2008).

Macrophages, characterized by the spectrum between classical M1 and M2 phenotype, also bear glycan-binding receptors, such as mannose receptor (MR) and DC-SIGN. Interestingly, the role of these CTLs in macrophages is still very broad, strongly relying on the context of inflammation. For instance, in the context of germinal centre formation, mannosylated IgM B cell receptor seems to promote the activation of B cell receptor (Amin et al. 2015). On the other hand, in a context of *Mycobacterium tuberculosis* infection, DC-SIGN-mediated recognition of bacterial glycans seems to induce an anti-inflammatory polarization in macrophages (Lugo-Villarino et al. 2018).

13.2.2 Role of Glycans in Adaptive Immune Cells Functions

Initially, the adaptive immune response relies on the ability to differentiate self-antigens derived from pathogens/damage host cells. This process is orchestrated by antigen-presenting cells (APCs), which display antigens attached to major histocompatibility complexes (MHCs) that are presented to T cells (den Haan et al. 2014; Lee et al. 2020). The MHC is a family of structurally and genetically related glycoproteins that are able to control immune response through T cell activation (Neefjes et al. 2011). It encompasses two major classes of MHCs: the MHC class I (MHC-I), which can be expressed by all nucleated cells and reacts to intracellular bacteria, viral infections and cellular transformation (Hewitt 2003; Comber and Philip 2014); and the MHC class II (MHC-II), which responds to exogenous proteins (Storni and Bachmann 2004). MHC-II expression is limited to professional APCs, such as DCs, macrophages and B cells (Roche and Furuta 2015).

The glycosylation of proteins and receptors that participate in adaptive immune response, such as MHC molecules and TCRs, is essential for correct protein folding (Trombetta and Helenius 1998) to protect protein backbone from proteolysis (Wang et al. 2001) and to ensure a suitable distance between receptors and other molecules at the cell surface, in order to facilitate interactions (Grigorian et al. 2009). Indeed, it was already described that the blockade of MHC1a *N*-glycosylation leads to a severe increase in intracellular misfolded protein content and an impairment in cell surface expression and peptide presentation (Barbosa et al. 1987).

MHC-II molecules are constituted by two α and two β chains, each of which contains a transmembrane domain, contrasting with MHC-I molecules, which display only one β chain. The glycan composition of both chains within MHC-II also differs, with the α chain displaying

predominantly *N*-linked high-mannose and complex *N*-glycans and the β chain being composed by complex *N*-glycans (Unanue et al. 2016). MHC-I exhibits one single conserved site for *N*-glycosylation at Asn86, whereas MHC-II has three highly conserved *N*-glycosylation sites, two on the α chain (Asn78 and Asn118) and one on the β chain (Asn19) (Gauthier et al. 1998; Ryan and Cobb 2012). The *N*-glycan site on MHC-I is important for antigen binding to occur due to its role in recruiting chaperones that are involved in peptide loading (68). Glycoprotein modifications of MHC molecules are able to mediate immune responses. Ostankovitch and colleagues have described that the presentation of an MHC-I restricted epitope, derived from the membrane protein tyrosinase, requires retrotranslocation of glycosylated molecules from the endoplasmic reticulum to the cytosol. In particular, they have shown that proteasomes degrade tyrosinase molecules that are glycosylated and generate intermediate molecules that are not found in degradation of non-glycosylated molecules. In this context, the authors suggest that the glycosylation of these intermediate molecules influences their processing by the proteasome, stating a relevant role for glycosylation for the presentation of an MHC-I-restricted epitope derived from tyrosinase (Ostankovitch et al. 2009).

Besides presentation of glycan antigens to T cells, the glycosylation of MHC-II is intricately associated with T cell response also by modulating antigen binding. Accordingly, in APCs deficient for *Mgat2* glycogene it was demonstrated that the lack of complex *N*-glycans was detrimental for glycan antigen presentation by MHC-II and led to loss of T-cell activity (Ryan et al. 2011).

As mentioned above, T cell development, growth and differentiation are regulated by *N*-glycans and *O*-glycans (see Sect.13.1.1). The role of glycans in these cells has been demonstrated in several studies on different autoimmune disorders and also murine models of inflammation-associated diseases (Mkhikian et al. 2011; Dias et al. 2018a; Verhelst et al. 2020). Foremost, over the past 20 years, we have witnessed how critical are *N*-glycosylation alterations in regulating adaptive immunity, namely by mediating the T cell function, not only through the regulation of its primordial receptor, the TCR, but also its partner receptors (namely CTLA-4, CD45, CD25, CD28) (Pereira et al. 2018a, b).

An altered glycosylation of the TCR, namely reduced expression of complex branched *N*-glycans, has been described to lower the threshold for T cell activation, further modulating the surface expression of important growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (Demetriou et al. 2001; Morgan et al. 2004; Grigorian et al. 2007). In fact, one decade ago it was demonstrated that TCR *N*-glycosylation is regulated by TCR signalling. More precisely, it upregulates *N*-acetylglucosaminyltransferase V (GnT-V) and Golgi α-mannosidase enzymes at the mRNA level, in a synchronous manner, to enhance complex branching *N*-glycans branching in activated T cells, to avoid hyperactivation (Chen et al. 2009a).

Similarly, an increase of branched *N*-glycans on CD25 has been associated with increased cell surface retention with impact in the regulation of T cell differentiation and immune tolerance. It was demonstrated that by reducing UDP-GlcNAc (substrate for the initiation of branching in *N*-glycans) intracellular availability or the expression of branching glycosyltransferase, there is a decrease of CD25 surface retention and IL-2signalling, which promotes T helper-17 (Th17) over induced regulatory T cell (iTreg) differentiation (Araujo et al. 2017). Additionally, the function of CD28 co-stimulatory receptor has been shown to be mediated by *N*-glycosylation which can negatively regulate the interaction between CD28/CD80. Different approaches inhibiting *N*-glycosylation (in vitro *by* site mutagenesis on the five *N*-glycosylation sites in the extracellular domain and by enzymatic inhibitors of biosynthesis of *N*-linked oligosaccharide structures) resulted in a defective CD28 glycosylation and enhancement of the binding to CD80 expressed on APCs (Ma et al. 2004). Later, other study demonstrates that the high levels of polylactosamine in CD8 are on *N*-glycans, further suggesting polylactosamine structures on CD28 as critical players in regulating T cell activation

(Togayachi et al. 2007). Moreover, in 2011, another study has also shown that IL-2 and IL-7 have distinct effects in resting and activate T cells. Early by lowering branching *N*-glycans and TCR activation thresholds, these cytokines enhance T cell growth. Later, they promote self-tolerance by enhancing branching and CTLA-4 surface retention (Mkhikian et al. 2011).

Additionally, galectins are at the crossroad of tolerance and inflammation (Modenutti et al. 2019). Different members of the galectin family exhibit a 'double-edge sword' effect, acting either as negative or positive mediators of T cell homeostasis. Galectin-1, -2, -3 act as inhibitors of inflammation and T cell activity. One of the major regulations of T cell function by galectins is related to Gal-1's ability to negatively regulate Th1 and Th17 effector cells by inducing cell death. Interestingly, it was also shown that Th2 cells upregulate the expression of terminal α2,6-sialic acids, which inhibit Gal-1 binding, render a Gal-1-induced-apoptosis resistance by these cells (Toscano et al. 2007). Gal-3 also plays a pivotal role in the regulation of T cell activity, as it can constrict TCR clustering, by the binding to complex branched *N*-glycans of these receptors, generating lattice formation, controlling the threshold of T cell activation (Demetriou et al. 2001; Chen et al. 2009b). Moreover, Gal-2 also exhibits a suppressive effect by inducing apoptosis of *lamina propria* T lymphocytes attenuating acute and chronic mouse colitis (Paclik et al. 2008). In contrast, Gal-8 and -4 act as promoters of T cell activation, as it was described that Gal-8 binds to T cells through unique interactions with CD45 and promotes T-cell proliferation (Tribulatti et al. 2009), and that Gal-4 mediates $CD4^+$T cell stimulation, through IL-6 production, leading to exacerbation of T cell-mediated chronic colitis (Hokama et al. 2004).

The dual impact of Gal-9 on inflammation is still controversial since most studies have been conducted only using exogenous Gal-9 and the endogenous counterpart has been overlooked. In fact, exogenous Gal-9-based approaches have suggested it as a immunoregulator of T cell function and a suppressor of immune disease in vivo (Zhu et al. 2005; Madireddi et al. 2014; Wu et al. 2014). However, a very recent study has elucidated the role of endogenous Gal-9, being crucial for Th17 differentiation and T cell proliferation. Moreover, the same work described that high levels of Gal-9 in $CD4^+$ T cells isolated from PBMCs of multiple sclerosis patients are positively correlated with disease severity, highlighting its potential value as biomarker for autoimmune diseases (Chen et al. 2020).

Overall, galectins have a master role in the regulation of inflammation but one of the main questions remains to be elucidated: What are the precise mechanisms involved in the anti-inflammatory and immunoregulatory effects of different members of the galectin family?

13.3 Glycans in Chronic Inflammatory Diseases

13.3.1 Role of Glycans in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic debilitating disorder from the gastrointestinal tract comprising Crohn's disease (CD) and ulcerative colitis (UC). The etiopathogenesis of IBD is influenced by a complex interaction between environmental and genetics factors, microbiome and/or host immune response (Kaplan and Ng 2017). Its incidence has been increasing worldwide, including in paediatric populations (Ruel et al. 2014; Kaplan and Ng 2017).

Over the past decade, several studies on IBD pathogenesis have unveiled the fundamental role of glycans in the regulation of innate and adaptive immune responses associated with IBD development and progression (as reviewed in detail in (Dias et al. 2018a, b)).

13.3.1.1 Role of Glycans in the Regulation of Innate Immune Response and Gut Microbiome in IBD

The intestinal epithelial barrier represents the largest interface between the internal organs and the environment. Placed at the cell surface of

enterocytes, heavily glycosylated membrane mucins constitute the intestinal glycocalyx, which is the first line of defence against microbial translocation. This dense microbial community exerts a significant impact on intestinal physiology due to their ability to modulate immune development and to inhibit pathogen colonization (Hooper and Gordon 2001). Indeed, it poses an enormous challenge to the immune system, particularly for innate cells, since it needs to properly respond to pathogens without mounting an inflammatory response that may be detrimental to commensal microbes and may trigger the development of spontaneous inflammation.

Although innate immune cells are essential regulators of a healthy intestinal environment, not much is known about how glycosylation alterations dictate innate cell functions. Intestinal macrophages are one of the most represented populations of leukocytes in the intestine, which makes them first-aid players to maintain intestinal homeostasis (Bain and Mowat 2014). As it was discussed above for general inflammatory processes, alterations in glycosylation pathways were described to influence this innate cell population in the context of IBD. Shinzaki et al. have demonstrated, using a transgenic mouse model of GnT-V overexpression, that increased expression of complex branched *N*-glycans leads to increased colitis severity by inducing macrophage dysfunction and subsequently enhancing colorectal tumorigenesis (Shinzaki et al. 2016). Moreover, intestinal epithelial cell–specific deficiency of core 1–derived *O*-glycans in mice is associated with development of spontaneous colitis, inducing exacerbated infiltration by TNF-producing myeloid cells in colon mucosa (Fu et al. 2011; Nakayama et al. 2019).

Innate lymphoid cells (ILCs) display a relevant role in the establishment of intestinal homeostasis, since they are highly responsive to microbial stimulation (Vivier et al. 2018). In particular, group 3 ILCs (ILC3) are considerably abundant in mucosal tissues, being particularly involved in epithelial barrier integrity through the production of IL-17, IL-22 and GM-CSF (Neill and Flynn 2018). ILC3 are also able to modulate the intestinal glycocalyx via production of IL-22, which induces the upregulation of fucosyltransferase 2 (FUT2) mRNA on intestinal epithelial cells that in turn lead a protective effect against pathogens through the stabilization of commensal gut microbiota (Goto et al. 2014).

Glycans can act as major sources of energy for the microbiota (Koropatkin et al. 2012). Besides being able to utilize glycans derived from the diet, several bacteria can degrade *O*-linked glycans present in the epithelial mucus layer or *N*-linked glycans shed by epithelial cells to use them as nutrient source (Tailford et al. 2015; Ravcheev and Thiele 2017). Specific deletion of core 1–derived *O*-glycans on gut epithelial cells, using an IEC conditional mouse model lacking *C1galt1* specifically in intestinal epithelial cells, was shown to induce spontaneous colitis in mice (Fu et al. 2011). To clearly demonstrate that the loss of intestinal epithelial core 1–derived *O*-glycans is at the basis of colitis development in adult mice, the authors crossed *C1galt1*$^{f/f}$ mice with VillinCre-ER^{T2} transgenic mice, creating an inducible model of deficiency of intestinal epithelial *O*-glycans that developed colitis 5 days after induction with tamoxifen (Shinzaki et al. 2016). Indeed, alterations in the *O*-glycosylation profile of mucin-2 are described in UC patients with active disease, with an increment of smaller glycans and a decrease of complex glycans, and it associates with increased intestinal inflammation (Larsson et al. 2011), stating the crucial role of glycosylation alterations in intestinal epithelial barrier function.

13.3.1.2 Role of Glycans in the Regulation of Adaptive Immune Response in IBD

The disruption of gut mucosal barrier leads to a cascade of events starting with innate immune response, which initiates and drives a subsequent adaptive immune response within the colon *lamina propria*. This second line of defence involves mainly the activation of Th1, Th2, Th17 cells and suppression of the activity of Treg cells (Iwasaki and Medzhitov 2010). Nevertheless, in inflamed intestinal mucosa (in CD but not in UC or healthy controls), there is a unique subset of FoxP3^{+} T

cells that produce IL17 (the so-called Treg/Th17 axis) (Hovhannisyan et al. 2011). The origin of this distinct cell population is not fully understood, but it is postulated to be shaped by gut microbiome, despite the precise mechanism(s) underlying it remains unclear (Omenetti and Pizarro 2015).

Several studies have been shown that the T cell differentiation can be influenced by *N*-glycosylation alterations, both in mouse and human cells (Araujo et al. 2017; Dias et al. 2018a, b). Interestingly, in UC patients with active disease and also murine models of IBD (DSS-induced colitis), characterized by highly activated Th1 and Th17 immune response, it was demonstrated that T lymphocytes of *lamina propria* are deficient in complex branched *N*- glycans (codified by *MGAT5*) on the TCR (Dias et al. 2014). However, when this deficient mechanism is repaired by in vitro glycan supplementation of patient-derived colonic T cells, both Th1 and Th17 responses are diminished through reduction of respective pro-inflammatory cytokines production (TNF-α, IFN-γ and IL17A) and transcription factors at mRNA level (T-bet and RORγT). Moreover, in vivo studies showed that disease severity and progression were attenuated upon the restore of TCR branched *N*-glycosylation (Dias et al. 2018a, b). Similar impact was observed in the absence of core fucose *N*-glycans (codified by FUT8) which are highly expressed in T cells from patients with active IBD. Regarding the impact of glycans in Treg population, there are no significant alterations in IBD models (Dias et al. 2018a, b). Accordingly, in mouse and human cells, it was demonstrated that Treg suppressive function correlates with glycan expression levels, as Tregs with high expression of tri/tetra-antennary complex *N*-glycans present an enhanced ability to suppress $CD4^+$ and $CD8^+$ T cell proliferation (Cabral et al. 2017). However, these findings still impose more comprehensive studies about the glycophenotype of Treg subset, using realistic in vivo disease models and in clinical settings.

More recently, in two independent European cohorts of UC patients, specific genetic variants of *MGAT5* were shown to have a functional impact in the modulation of T cell glycosylation and plasma IgG glycome, being associated with worse disease course (Pereira et al. 2020). Briefly, UC patients display *MGAT5* single-nucleotide polymorphisms (SNPs) that are functionally associated with low transcription levels of *MGAT5* glycogene in colonic and circulating T cells and with agalactosylation of IgGs (a pro-inflammatory glycophenotype of IgG, observed in other autoimmune disorders (Ercan et al. 2010; Vučković et al. 2015; Momozawa et al. 2018)). Importantly, despite these evidences suggesting *MGAT5* gene as a common driver that simultaneously regulates the function and activity of both humoral and adaptive components involved in IBD development, it is not clarified yet whether the alterations on IgG glycoprofile are or not a T-cell-dependent mechanism.

Overall, glycans are master regulators of intestinal homeostasis and inflammation as they play a role on driving a fine-tuned dynamic between intestinal epithelial barrier function, the immune system and the gut microbiome. Nevertheless, further studies focused on the glycobiome are needed to fully understand the regulatory mechanisms associated with IBD.

13.3.2 Role of Glycans in Glomerulonephritis

Glomerulonephritis is a general inflammation of a specific portion of the kidney—glomeruli. Glomeruli represent an essential functional unit of the kidney, composed by podocytes organized together to build a filtration barrier. This barrier is highly regulated by the ability of podocytes of contracting and stretching, therefore controlling the passage of water or proteins/compounds (Petrosyan et al. 2019).

Inflammation of glomeruli can occur as an acute event which can be resolved within days, or be prolonged through months/years, precluding in a chronic inflammation with severe damage to the kidney function. The etiopathogenesis of glomerulonephritis is still to be elucidated, however it can be associated with autoimmune disorders (such as SLE or IgA nephropathy) as well as with

an aggravation and extension of a primary acute inflammation, such as drug- or infection-induced (Webster and Pusey 2017). In both scenarios, it is important to account for two different compartments which can be dysregulated: both immune infiltration, which seems to be over-activated and instructing a cytotoxic immune response against podocytes and mesangial cells in the glomeruli, as well as the non-immune compartment, which could be triggering an aberrant immune recruitment. Glycosylation plays a crucial role in both compartment perspectives, giving rise to new hypotheses in the etiopathogenesis of glomerulonephritis.

GnT-V (as mentioned before) is the enzyme responsible for the addition of β1,6-branched GlcNAc, allowing the following extension of poly-*N*-acetyl-lactosamine (Gal-β1,4-GlcNAc) group. This group is a binding motif for Gal-3 lattice, allowing a spatial distance between TCRs, preventing TCR clustering and activation. Hereupon, Demetriou et al. showed that at 12–20 months of age, *Mgat5* null mice appear to exhibit signs of glomerulonephritis (Demetriou et al. 2001). This glomerulonephritis was characterized by immune infiltration in the glomeruli and mesangial area, as well as severe glomeruli destruction; phenotypically most severe cases of glomerulonephritis (32%) showed haematuria, proteinuria and a characteristic crescentic glomerulonephritis with fibrosis in the Bowman capsule. The hyperactivation observed in *Mgat5*$^{-/-}$ could be instructing a stronger immune response in the kidney, as it does in the colon (Demetriou et al. 2001; Dias et al. 2018a, b).

Interestingly, Chui et al. have observed that murine kidney glycoproteins are highly dependent on α-mannosidase II for the correct glycan repertoire, a special feature which was not observed in other tissues, since an alternative form of α-mannosidase II takes its role. This observation reveals the importance of α-mannosidase II enzyme kidney-associated inflammation. Accordingly, these mice develop a severe autoimmune-associated glomerulonephritis at 12 months of age, displaying high levels of autoantibodies, with IgG, IgM, IgA and C3 complement deposition on glomeruli, as well as plasma cells and neutrophil infiltration (Chui et al. 2001).

In both mice models, it is possible to assume that non-immune compartment is also playing a role, since MGAT5 or MAN2A1 are absent in all organism cells/tissues. Given that, an interesting library of C-type lectins has been studied in the scope of immune cell recognition, not only in infection models, but also in inflammatory diseases, since aberrant glycans are being exposed at the surface of supposedly healthy cells. It was interesting to observe that glomerulonephritis was attenuated in mice after treatment with anti-DC-SIGN antibody (Cai et al. 2016). Moreover, mannose-binding lectin (MBL), a soluble c-type lectin which recognizes agalactosylated glycoproteins, was detected in the glomerulonephritis kidney biopsies of lupus patients, with no changes in the serum levels (Lhotta et al. 1999). Altogether, there is a body of evidence that argues a possible role in the aberrant glycoprofile of non-immune compartment for the immune response triggering.

Despite the lack of knowledge regarding the role of glycosylation in SLE, recent findings have been describing a deficiency in complex *N*-glycans at the surface of kidney epithelial compartment (Alves et al. 2020). This altered glycoprofile appears to be associated with the promotion of a chronic inflammatory response, however more studies are needed to validate these observations.

13.3.3 Role of Glycans in Myopathies

Idiopathic inflammatory myopathies (IIM) are a group of rare diseases of autoimmune nature, whose etiopathogenesis is far from being totally understood. Factors implicated in autoimmune diseases in general, namely immune innate and adaptive dysregulations associated with non-immune mechanisms, have been implicated, but how they interplay to give rise to the diverse pathogenic phenotypes remains elusive (Miller et al. 2018). Muscle cells surface is enriched in glycoproteins, either alone or in glycoprotein complexes, and several lines of evidence provide

support for a fundamental role of glycosylation in muscle homeostasis and function (Broccolini et al. 2009; Townsend 2014; McMorran et al. 2016). Hereditary inclusion-body myositis (hIBM) bears a muscle phenotype that resembles in most aspects sporadic inclusion-body myositis (sIBM) (one of the IIM subgroups) and is associated with mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) gene. GNE codes for an enzyme expressed in several tissues and it is critical for the biosynthesis of sialic acid. GNE mutations result in glycosylation changes, namely hyposialylation of muscle glycoproteins and prophylactic supplementation of a sialic acid precursor (*N*-acetylmannosamine (ManNAc) was shown to prevent the muscle phenotype in mice with gene mutations that cause hIBM (Malicdan et al. 2009). Changes in muscle glycosylation in human disease have focused in muscular dystrophies and congenital glycosylation disorders, but recent studies have shown that muscle cell surface glycosylation is finely regulated and subjected to alterations under inflammatory condition (Wiendl et al. 2005), pointing to an interaction between muscle glycocalyx and the extracellular milieu, which is particularly enriched in immune cells and antibodies in IIM patients (Afzali et al. 2018).

Changes in the signature of healthy muscle cells could be indeed associated with immune infiltration and development of a dysregulated response in a tissue-target manner. Understanding the role of *N*-glycans, either at the immune compartment as well as in the epithelial component may contribute to a better knowledge of the etiopathogenesis of inflammatory myopathies.

13.4 Glycans as Potential Targets for Diagnosis, Prognosis and Therapy in Chronic Inflammatory Diseases

Chronic Inflammatory Diseases including IBD, SLE and IIM present a big challenge for the correct diagnosis and are characterized by a large variation in response to treatment and issues in predicting outcomes to conventional therapies (Podolsky 2002; Dias et al. 2018a, b; Verhelst et al. 2020). Early diagnosis and prognosis of these types of diseases are critical to decrease morbidity, improve quality of life and decrease disability of patients, since the delayed initiation of appropriate therapy may contribute to unsuccessful outcomes. Therefore, there is a clinical need to develop better diagnostics, more effective and preventive approaches. The identification of novel biomarkers of disease whose exploitation may represent a promising novel therapeutic strategy for inflammatory diseases will allow the selection of patients according to their proneness to develop aggressive/complicated disease course and consequently an adequate redirection of therapy (Pereira et al. 2018a, b).

Due to their role in cell functions, glycans have recently been appreciated as a crucial factor regulated in pathologic events leading to development of immune-mediated diseases. In fact, the relationship between glycosylation alterations and its functional impact in the etiopathogenesis of many autoimmune diseases is a new and promising field for the development of novel therapies directed to improve individualized therapy and to develop better diagnostic and prognostic approaches (Dias et al. 2018a, b; Hanić et al. 2019; Verhelst et al. 2020). Lower levels of branched *N*-glycans in colon biopsies diagnosis predict patients that do not respond to standard therapy with 75% specificity, whereas patients presenting high levels of branched *N*-glycans display a favourable therapeutic and disease outcome. Interestingly, this glyco-biomarker combined with the analysis of C-reactive protein (CRP), a clinical biomarker used as a predictor of inflammation, constitutes a powerful tool with improved prognostic capacity. IBD patients with low branched *N*-glycans and high CRP levels at diagnosis early require an aggressive and non-conventional therapy (Pereira et al. 2018a, b).

Additionally, recent discoveries on the role of plasma glycoproteins have pushed forward the expanding area of GlycoMedicine to the forefront for many clinical applications (Theodoratou et al. 2014; Hanić et al. 2019; Reily et al. 2019). Highly inflammatory glycosylation signature of plasma immunoglobulin G (IgG) has been asso-

ciated as clinical features of IBD and SLE (Arnold et al. 2007; Šimurina et al. 2018). In fact, a pronounced decreased galactosylation of IgG-Fc is observed in SLE and IBD patients, representing in turn a great indicator of chronic inflammation with significant diagnostic value. Moreover, a decrease of di-galactosylated IgG *N*-glycans in IBD patients compared with healthy controls was already reported (Shinzaki et al. 2008). The role of glycans as diagnostic and prognostic IBD biomarkers was further illustrated by *N*-glycan analysis of total plasma proteins where an increase in glycan branching, a decreased abundance of hybrid and high-mannose structures, higher total sialylation, and lower fucosylation were observed in IBD patients compared with healthy individuals (Clerc et al. 2018). Likewise, some reports have shown that the glycosignature of IgG changes between patients with UC and CD, revealing differences on level of fucosylation, galactosylation, and bisection (Trbojević Akmačić et al. 2015; Šimurina et al. 2018). A higher α2,3-linked sialylation and higher bisection of plasma glycoproteins were detected in CD compared with UC (Clerc et al. 2018). Interestingly, increased agalactosylation (loss of a terminal galactose) of IgGs serum levels displayed by patients with CD is correlated with levels of CRP and associated as well with more extensive and progressive disease (Dubé et al. 1990). Thus, IgG Fc-galactosylation seems to be a relevant biomarker for the prognosis of IBD.

Another glycobiomarker for IBD is glycoprotein acetylation (GlycA). Higher complex *N*-glycans expression in acute-phase glycoproteins (such as haptoglobin, α-1-acid glycoprotein, transferrin and α-1-antichymotrypsin) has been associated with worse disease severity in SLE (Connelly et al. 2017; Dierckx et al. 2019).

The exploration of pathogenic role of glycans variation in IBD is also extended to the expression of glycan receptors. Studies demonstrated that differential expression of glycan receptors, such as galectins, play a major influence on the IBD development and the serum galectins can be used as potential biomarkers of IBD and disease activity (Yu et al. 2020). As already mentioned along the chapter, circulating galectins are usually altered in disease context. L-selectin, a cell adhesion molecule of lymphocytes that recognizes endothelial ligands, was found to be increased in serum samples of UC patients compared with healthy controls (Seidelin et al. 1998). Patients with active IBD showed higher serum levels of galectin-1 and -3 comparatively with healthy individuals (Frol'ová et al. 2009). Galectin-1 discriminated IBD from healthy individuals with 71% sensitivity and 87% specificity (Yu et al. 2020); in turn galectin-3 discriminated IBD from healthy controls with 53% sensitivity and 87% specificity (Yu et al. 2020). Hence, galectins might be useful as a powerful biomarker for the diagnosis of IBD.

It has been reported the contribution of genetic variants of key glycoenzymes has an important role in regulating susceptibility and etiopathogenesis in chronic inflammatory diseases (Podolsky 2002; Dias et al. 2018a, b). Recently, a novel genetic risk locus was identified including intronic SNPs in the glycogene MGAT5 that are functionally correlated with glycosylation alterations on T cells and on plasma IgGs and that presented strong association with clinical severity/complication of the UC disease (Dias et al. 2018a, b; Pereira et al. 2020). The rs3814022 and rs4953911 were found to be significantly correlated with lower levels of Fc domain monogalactosylation of IgG2 and IgG3. The rs4953911 was also found to be associated with agalactosylation of IgG1 of which it is associated with induction of a proinflammatory effector functions. Individuals with genetic variations on FUT2 are known to present increased susceptibility to develop IBD (Rausch et al. 2011; Lewis et al. 2015).

Additional biomarkers used into diagnostic of autoimmune and inflammatory disorders are the serum antibodies against glycans (Zhou et al. 2016). Anti-glycoprotein 2, anti-mannobioside carbohydrate IgG (Li et al. 2008; Bogdanos et al. 2011) and anti-*Saccharomyces cerevisiae* are some of the anti-glycan antibodies (Annese et al. 2004) used to perform the diagnosis of IBD and

differentiate UC from CD with a prognostic value (prediction of aggressive disease course). These observations pinpointed the role of glycosylation patterns, such as serum glycome, as a potential diagnostic and prognostic tool among different inflammatory disorders.

Recent discoveries on the role of glycans as a powerful translational therapy have pushed forward the expanding area of glycotherapy to the forefront of in clinical context.

The application of carbohydrate-recognizing receptor inhibitors as a pharmacological tool to block target-pathogenic processes is an example of new generation of therapeutics. Pharmacological blockade of selectins by anti-selectin monoclonal antibodies has gained special interest as a therapy to IBD. Natalizumab (an α4-integrin antagonist) (Gordon et al. 2001, 2002) and vedolizumab (which selectively blocks trafficking of α4β7-positive lymphocytes to the gut) (Feagan et al. 2013; Sandborn et al. 2013) have been clinically applicated to treat IBD. Moreover, the administration of glycoengineered therapeutic monoclonal antibodies, as deglycosylated antibodies (treated with endoglycosidase S), were found to hamper the formation of immune complexes, reducing pathology of disease in case of SLE (Lood et al. 2012).

Interestingly, the potential of glycans supplementation has been described as a promising adjuvant therapy, namely GlcNAc for patients with UC. The metabolic GlcNAc supplementation of mucosal T cells isolated from patients with active UC improved branched *N*-glycosylation on the T cell receptor, consequently controlling T cell activation and function (Dias et al. 2018a, b). Remarkably, a pilot study of oral supplementation with GlcNAc in paediatric IBD reveals a potential role of GlcNAc as a powerful therapeutic agent. More than half of children under GlcNAc treatment exhibited clinical remission with evidence of histological improvement. Moreover, the properties of GlcNAc as a therapy were already verified in a pilot study of paediatric patients with IBD (Salvatore et al. 2000).

13.5 Concluding Remarks/ Conclusion

The relevance of glycans in the study of inflammatory diseases extends from the intrinsic effects on immune cells as well as the correct recognition of danger glycosylation patterns. From immune cells' correct development through the migration and extravasation to the inflamed tissues, glycosylation plays an important role. Moreover, specific glycan switches seem to contribute to the regulation and/or dysregulation of the immune cell response.

Various advances on the field of glycosylation have been contributing to a paradigm shift in the patients' stratification, enabling a personalized medicine through optimized preventive and improving prognostic accuracy in the clinical management of patients. Moreover, the identification of glycosylation unbalance into inflammatory diseases' pathophysiology constitutes an opportunity to improve the target-specific therapy of patients without side effects and with a low cost.

Compliance with Ethical Standards

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Protein Glycosylation in Diabetes

14

Tamara Štambuk and Olga Gornik

Abstract

Diabetes mellitus is a group of metabolic disorders characterized by the presence of hyperglycaemia. Due to its high prevalence and substantial heterogeneity, many studies have been investigating markers that could identify predisposition for the disease development, differentiate between the various subtypes, establish early diagnosis, predict complications or represent novel therapeutic targets. *N*-glycans, complex oligosaccharide molecules covalently linked to proteins, emerged as potential markers and functional effectors of various diabetes subtypes, appearing to have the capacity to meet these requirements. For instance, it has been shown that *N*-glycome changes in patients with type 2 diabetes and that *N*-glycans can even identify individuals with an increased risk for its development. Moreover, genome-wide association studies identified glycosyltransferase genes as candidate causal genes for both type 1 and type 2 diabetes. *N*-glycans have also been suggested to have a major role in preventing the impairment of glucose-stimulated insulin secretion by modulating cell surface expression of glucose transporters. In this chapter we aimed to describe four major diabetes subtypes: type 1, type 2, gestational and monogenic diabetes, giving an overview of suggested role for *N*-glycosylation in their development, diagnosis and management.

Keywords

Diabetes mellitus · *N*-glycans · Protein glycosylation · Diabetes biomarkers · Diabetes diagnosis

14.1 Diabetes Mellitus

Diabetes mellitus (later in this chapter referred as diabetes) is a group of metabolic disorders characterized by the presence of hyperglycaemia in the absence of treatment due to incapability of the body to produce enough insulin by pancreas (type 1 diabetes mellitus), inability to use it (type 2 diabetes mellitus), or occur as a temporary condition (gestational diabetes during pregnancy—GDM) (The American Diabetes Association 2020). The disease burden related to diabetes is very high and rising in every country. This does not solely come from the fact that over 400 mil-

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lion people worldwide has diabetes and that 1.6 million deaths are directly attributed to this disease each year (Cho et al. 2018). Diabetes is a lifetime disease, with reduced life expectancy, premature morbidity and mortality. This disease can lead to multisystem microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (ischaemic heart disease, stroke) complications (Fowler 2011; Forouhi and Wareham 2014).

Since diabetes comprises many disorders characterized by hyperglycaemia, from the 1965 the World Health Organization (WHO) has periodically updated guidance on how to classify diabetes (Rodriguez et al. 2002). There are two major types: type 1 diabetes (T1DM) and type 2 diabetes (T2DM), differentiated by the age of onset, degree of loss of β-cell function, degree of insulin resistance, presence of disease associated antibodies and requirement for insulin treatment (The American Diabetes Association 2020). Nevertheless, nowadays it is clear that none of these characteristics clearly discriminates between these types which are becoming more alike due to increased prevalence of obesity in children (Pulgaron and Delamater 2014), occurrence of T1DM in older population (Dhaliwal and Weinstock 2014) or T2DM at young age (Alberti et al. 2004). Furthermore, due to development of modern analytical techniques, genetic tests (Stein et al. 2014) and increasing knowledge on the pathophysiology, scientists and clinicians identified many additional subtypes of diabetes, even with the implications for the choice of treatment in some cases. This is why in 2019 WHO published a new classification of diabetes with practical guidance for diagnosis and treatment of diabetes. They classified diabetes as: T1DM, T2DM, hybrid forms of diabetes, other specific types (including monogenic diabetes), unclassified diabetes and hyperglycaemia first detected during pregnancy. The main characteristics of the major subtypes are given in Fig. 14.1.

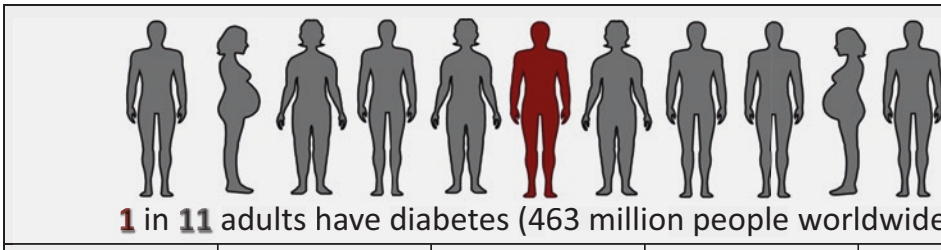
Currently, there are four diagnostic tests recommended for diabetes (International Expert Committee 2009): measurement of fasting plasma glucose, 2-h post-load plasma glucose after a 75 g oral glucose tolerance test (OGTT), haemoglobin A1c (HbA1c) and a random blood glucose combined with the presence of certain symptoms. Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dl), 2-h post-load plasma glucose ≥11.1 mmol/L (200 mg/dl), HbA1c ≥ 6.5% (48 mmol/mol), or a random blood glucose ≥11.1 mmol/L (200 mg/dl) in the presence of symptoms are considered as diabetes.

Differentiation of diabetes subtypes is mainly based on clinical parameters (The American Diabetes Association 2020). This approach can be improved with specific additional tests, but due to limited accessibility those would have limited global applicability. Autoantibodies directed to β-cell components (glutamic acid decarboxylase—GAD65, islet antigen-2—IA-2, zinc transporter 8—ZnT8 and insulin) are usually found in people with T1DM (Ziegler et al. 2013) but can also be found in a small number of people with T2DM. Endogenous insulin production, assessed by measuring blood or urine C-peptide, in the early stages of diabetes, provides information which may help to distinguish T1DM from T2DM (The American Diabetes Association 2020), but is not routinely done clinically. Genotyping is relevant to monogenic diabetes (Kleinberger and Pollin 2015; Althari and Gloyn 2015), but not to T1DM (DiMeglio et al. 2018) or T2DM (Vassy et al. 2012) which are polygenic, with over 100 genetic markers associated through different genome wide association studies.

Due to high prevalence of diabetes worldwide and number of different subtypes, many studies investigating novel biomarkers that could identify predisposition for the diabetes development or can be used to establish early diagnosis, predict complications and disease outcome, as well as differentiate between subtypes or identify novel medications and therapeutic targets are currently globally conducted.

14.2 Glycans as Biomarkers in Diabetes

Group of biomolecules, named glycans, also found themselves in the focus of a certain number of scientists who are trying to investigate



Classification	Type 1 diabetes	Type 2 diabetes	Specific types	Gestational diabetes
Prevalence	5–10% of diabetes	90 – 95% of diabetes	1.5 – 2% of diabetes	~ 16% of pregnancies (region-dependent range of 1-30%)
Age of onset	Six months – young adult	Adulthood	MODY – adolescence to young adulthood Neonatal – first 6 months of life	Second or third trimester of pregnancy
Genetic aethiology	Polygenic	Polygenic	MODY – monogenic, autosomal dominant Neonatal – monogenic in 85% of cases	Polygenic
Immune component	Autoimmune β-cell destruction	None	Rarely, the presence of autoantibodies can be detected	None
Insulin secretion	Usually – absolute insulin deficiency	Progressive loss of adequate β-cell insulin secretion	MODY – impaired insulin secretion Pancreatic diabetes – structural and functional loss of insulin secretion	Often indicative of underlying β-cell dysfunction
Insulin sensitivity	Normal when controlled	Peripherial insulin resistance	Normal (may be decreased in obesity)	Increased peripherial insulin resistance

Fig. 14.1 Basic distinctive features of major diabetes subtypes. Characteristics have been based on American Diabetes Association and International Diabetes Federation definitions and diabetes classification. MODY—maturity onset diabetes of the young

their role in the pathophysiology of diabetes, as well as evaluate their potential as biomarkers for the above-mentioned applications (Rudman et al. 2019).

Glycosylation is the most diverse protein modification by which complex glycan structures are added to protein or lipid backbones (Varki et al. 2015). Contrary to glycation, which has been usually connected to diabetes (Ulrich and Cerami 2001), glycosylation is a multi-stage enzymatic process regulated by a huge network of genes. This process results in branched complex structures made of monosaccharide units linked by chemical bonds. Proper glycosylation is important for correct protein folding, cell structure maintenance, receptor–ligand interactions, cell signalling, cell–cell recognition and immune defence and glycosylation changes are well known to influence protein functions (Varki et al. 2015). The great assortment of glycans results in numerous glycoforms of the same protein, giving a human proteome completely new dimension (Gornik et al. 2012).

A recent study showed that glycans are directly involved in the pathophysiology of many diseases and that the additional knowledge from glycoscience is needed to realize the goals of personalized medicine (National Research Council 2012). Contrary to genetic biomarkers, which are set for a lifetime, glycan biomarkers also reflect the physiological and pathological situation of an individual (Axford 2001). Thus, most of human diseases, ranging from infection to autoimmunity (Axford 2001) and including cancer (Munkley and Elliott 2016), are associated with glycosylation changes that often precede disease onset. Furthermore, as the structural diversity of glycans represents a complex pool of information (more than proteins, lipids, or nucleic acids), glycosylation changes are mostly specific for the disease and/or disease stage, making them valuable diagnostic and prognostic markers for complex pathologies (Singh et al. 2020; Poland et al. 2001).

N-glycosylation changes have been described in different diseases, including type 1 diabetes (Poland et al. 2001; Singh et al. 2020), type 2 diabetes (Keser et al. 2017), gestational diabetes (Lee et al. 2011) and HNF1A MODY (Juszczak et al. 2019). Glycans are thus being considered as biomarkers of ongoing pathological condition as well as evaluated as discriminating tools between diabetes subtypes. It was also implied that *N*-glycosylation of plasma proteins can even identify individuals at an increased risk of developing diabetes in the future (Keser et al. 2017). A notable discovery of aberrant *N*-glycosylation of pancreatic β-cell glucose transporter 2 (GLUT2) in type 2 diabetes that leads to impairment of insulin secretion and can be targeted to suppress diabetes provides a promising novel drug target (Ohtsubo et al. 2005, 2011, Ohtsubo et al. 2013).

Advances in laboratory technologies (Keser et al. 2017; Royle et al. 2008) enabled large scale studies that evaluate the effects of different genetic variants on glycosylation patterns and their association with disease mechanisms through the genome-wide association studies (GWASs) (Lauc et al. 2010; Wahl et al. 2018). These studies identified the same genes associated with *N*-glycosylation as those previously reported for diabetes.

This chapter will focus on the *N*-glycosylation in T1DM, T2DM, gestational diabetes and monogenic diabetes. Some of these changes have been related to diabetes onset, and some to diabetes complications. Also, there is an accumulating evidence implicating these changes in mechanism underlying diabetes development.

14.3 Hexosamine Biosynthetic Pathway (HBP) and Modification of Glycans

The importance of *N*-glycosylation and origin of its changes in diabetes is not clear, but it is known that variations in glucose metabolism directly affect glycosylation through hexosamine biosynthetic pathway (HBP).

The HBP is a branch of glycolysis responsible for the production of uridine diphosphate-*N*-

acetylglucosamine (UDP-GlcNAc) (de Queiroz et al. 2019). Its final product is considered an important nutrient sensor, since HBP uses as substrates molecules from carbohydrate (glucose), lipid (acetyl-CoA), amino acid (glutamine) and nucleotide (UTP) metabolism to generate UDP-GlcNAc. This metabolite is an activated monosaccharide used as substrate for glycosyltrasferases in glycosylation reactions (Aebi 2013), or to generate UDP-GlcNAc-derived activated monosaccharides, also used for glycosylation, such as UDP-*N*-acetylgalactosamine (UDP-GalNAc) and cytidine monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac).

Glucose-provoked tissue damage is, among other pathways, also assumed to be mediated through the HBP (Aebi 2013; McClain 2002). Under homeostatic conditions, around 3% of total glucose is utilized through this pathway (Marshall et al. 1991). However, under conditions of hyperglycaemia the percentage of total glucose utilized through the HBP could be enhanced, leading to increased levels of the major product of the HBP, UDP-GlcNAc. This was proposed as one of the mechanisms for the increase in highly branched *N*-glycan structures on human plasma proteins among the adult T1DM patients with kidney disease (Bermingham et al. 2018) and among T2DM population (Marshall et al. 1991) and population with the increased risk of development of this disease (Keser et al. 2017).

A majority of studies on UDP-GlcNAc availability and Golgi enzyme activity relation to production of branched glycans are focused on UDP-GlcNAc stimulation of *O*-glycosylation as the possible mechanism of diabetic complications (Brownlee 2001), but there is increasing evidence for the role of highly branched *N*-glycans in autoimmunity development, which is mediated by increase in UDP-GlcNAc availability through HBP pathway (Brownlee 2001; Yki-Järvinen et al. 1998). The proposed mechanism involves number of different glycoproteins present on T cells that regulate T cell activation, which is increased upon inhibition of *N*-glycosylation of those proteins.

14.4 Type 1 Diabetes Mellitus

T1DM is one of the most common chronic diseases in childhood and results from autoimmune destruction of β-cells responsible for the production of insulin in the pancreas. In terms of potential pathogenic mechanisms, CD8+ T-cells are the most predominant population within the insulitis lesion, followed by macrophages (CD68+), CD4+ T-cells, B-lymphocytes (CD20+) and plasma cells (CD138+) (The American Diabetes Association 2020). The disease is defined by the presence of one or more autoantibodies (Ziegler et al. 2013). However, in the minority of patients with T1DM there is an absence of pancreatic autoantibodies, and in some patients with clinical diagnosis of T2DM there is evidence of islet autoimmunity (Pozzilli and Guglielmi 2009). T1DM usually presents in childhood, but one-fourth of cases are diagnosed in the adult age. Polydipsia, polyphagia and polyuria, along with overt hyperglycaemia remain diagnostic hallmarks in children and adolescents and to a lesser extent in adults.

T1DM accounts for approximately two-thirds of new diagnoses of diabetes in patients ≤19 years of age, despite the increasing rate of T2DM. The incidence of childhood T1DM is rising worldwide, with reported increases of 2–5% per year (Patterson et al. 2009) and the reasons for this are unknown. The connection of the disease incidence and the geographical latitude has been observed (Liese et al. 2010). The fact that relocation of a person from a region of low to high incidence increases the risk for T1DM suggests a causative role for environmental factor(s). However, the risk of developing T1DM is also significantly increased in close relatives of a patient, what demonstrates the complexity and genetic basis of the pathogenesis of this disease. T1DM represents about 10–15% incidence in individuals who have first or second degree relative with diabetes but may also occur in individuals without any family history of diabetes. It was also shown that earlier diagnosis of T1DM associates with fewer complications at diagnosis and improved metabolic control in the upcoming years (Lundgren et al. 2014). Also, early treat-

ment has been shown vital in the prevention of the long-term complications.

In genetically susceptible individuals, exposure to one or more environmental agents appears to trigger an immune response that ultimately causes destruction of the insulin-producing pancreatic β-cells (Hassan et al. 2012). Identification of these factors should lead to a better understanding of the pathogenesis of the disease and aid in developing strategies to prevent T1DM. The increase in understanding of the pathogenesis of T1DM has made it possible to consider intervention to slow the autoimmune disease process in an attempt to delay or even prevent the onset of hyperglycaemia. Till now, no successful strategy for the prevention of T1DM has yet been identified and recognizing subjects who are at high risk of T1DM became an important task. Combinations of immune, genetic and metabolic markers are considered and used for this purpose, but any new insights and predictive markers would be of a great value. Genetic markers for T1DM are present from birth, immune markers are detectable after the onset of the autoimmune process, and metabolic markers can be detected with sensitive tests once enough β-cell damage has occurred, but before the onset of symptomatic hyperglycaemia (The American Diabetes Association 2020). This long latent period reflects the large number of functioning β cells that must be lost before hyperglycaemia occurs. Although there is no specific test to distinguish between the different subtypes of diabetes, T1DM is suggested by the presence of circulating specific autoantibodies. However, the absence of pancreatic autoantibodies does not rule out the possibility of T1DM and also up to 30% of individuals with the classical genetic appearance and presentation of T2DM have positive autoantibodies and may have a slowly progressive type of autoimmune diabetes (Pozzilli and Pieralice 2018). The rate of progression of the immune injury is highly variable, even among high-risk subjects who have one or more of the relevant serum autoantibodies. In some subjects, as an example, progression is so slow that diabetes does not occur for many years or perhaps ever.

More than 50 genetic loci have been connected with T1DM development (Morahan 2012), while the highest genetic susceptibility is mapped to major histocompatibility complex (MHC) region (Erlich et al. 2008). Among the implicated loci is fucosyltransferase 2 gene (*FUT2*), identified as causal candidate gene (Onengut-Gumuscu et al. 2015). This gene encodes a glycosyltransferase responsible for the addition of α1,2-linked fucose to the terminal galactose on different glycans and therefore, the formation of the H antigen and type O blood group; a precursor of the ABO histo-blood group antigens in body fluids and on the intestinal mucosa. Individuals homozygous for non-functional *FUT2* allele fail to present histo-blood group antigens in saliva and mucosal surfaces (termed as non-secretors) and those individuals that are heterozygous for one of two non-secretor variants are termed as secretors (Ferrer-Admetlla et al. 2009). Genetic study conducted on T1DM individuals associated the non-secretor genotype with the susceptibility to this disease; thus, linking the host resistance to infection with susceptibility to developing autoimmune disease (Smyth et al. 2011).

There is also evidence for the role of highly branched *N*-glycans in autoimmunity mediated by the alpha-1,6-mannosylglycoprotein 6-beta-*N*-acetylglucosaminyltransferase A (MGAT5). This enzyme is responsible for the formation of highly branched, tetraantennary *N*-glycans through extension of the 1–6 arm of the glycan core with GlcNAc residue, and thus produces an intermediate for subsequent addition of the *N*-acetyllactosamine (LacNAc), which is a ligand for galectins (Johannes et al. 2018). Loss of *MGAT5* expression lowers T-cell activation threshold (Demetriou et al. 2001). Clusters of a certain number of T-cell receptors (TCRs) at the antigen presentation site are required for T-cell activation (Johannes et al. 2018). It was demonstrated that MGAT5$^{-/-}$ T -cells have lower T-cell activation threshold due to enhancement of TCRs. Pretreatment of wild-type T-cells with lactose in order to compete for galectin binding resulted in TCR clustering; with conclusion that a galectin–glycoprotein lattice supported by

MGAT5-synthesized *N*-glycans limits TCR recruitment to the site of antigen presentation. In vivo, Mgat5$^{-/-}$ mice exhibited several autoimmune phenotypes. Another study showed that galectin-1 induced apoptosis of activated human T-cells was decreased when *N*-glycosylation was inhibited following the treatment of cells by swainsonine, inhibitor of α-mannosidase II, an enzyme upstream of MGAT5 in *N*-glycosylation pathway (Dennis 1986). In addition to TCRs, co-stimulatory molecules, such as a glycoprotein CD28, are required for a productive immune response. It was shown that CD28 ligation lowers the threshold for T-cell activation (Michel et al. 2001). Also, another glycoprotein with a major role in the process of T-cell activation has been identified on T-cells, the cytotoxic T-lymphocyte antigen-4 (CTLA-4), an inhibitory molecule in the aforementioned process. *CTLA-4* is identified as causal candidate gene important in T1DM pathogenesis (Nisticò et al. 1996).

Studies have demonstrated that antibodies against IFN-γ administrated to mice prevented the induction of diabetes (Debray-Sachs et al. 1991). Later studies showed that IFN-γR might play a main role in CD4^{+} T-cell mediated β-cell damage; however not in CD8^{+} T-cell mediated (Yi et al. 2012). Moreover, IL-4 was shown to prevent insulitis and diabetes development; again using NOD mice as models (Cameron et al. 1997). Oral GlcNAc supplementation in these mice enhanced T-cell *N*-glycan branching and protected against the disease (Grigorian et al. 2007). IL-2Rα (CD25) is also an *N*-glycoprotein involved in T-cell survival and proliferation; its *N*-glycosylation and thus also a downstream signalling was inhibited by supplementation with glucosamine; thus demonstrating a dual role of *N*-glycosylation in T-cell differentiation (Chien et al. 2015).

In our recent study we have also shown that serum *N*-glycosylation changes in adult T1DM population are correlated with diabetic complications (Bermingham et al. 2018). It was demonstrated that *N*-glycan profile of both total serum proteins and of immunoglobulin G (IgG) is altered in adult T1DM patients with kidney disease. The most important observation was increase in complex *N*-glycans (highly branched, triantennary and tetraantennary structures) and decrease in simpler biantennary *N*-glycans among total serum proteins that was correlated with higher HbA1c, higher albumin-to-creatinine ratio (ACR) and steeper decline in estimated glomerular filtration rate (eGFR), reflecting poorer glycaemic control and renal function. The most complex IgG *N*-glycan is a biantennary *N*-glycan, however, again increase in more complex (more galactosylated and sialylated biantennary structures) and decrease in simpler (monogalactosylated biantennary) *N*-glycans was observed, and correlated with higher HbA1c, higher ACR and greater mean annual decline in eGFR.

14.5 Type 2 Diabetes Mellitus

T2DM represents an expanding global health problem, as its prevalence steadily increases and it is estimated to nearly double by the year 2035 (DeFronzo et al. 2015). It is by far the most frequent form of diabetes, accounting for 90–95% of cases (The American Diabetes Association 2020). T2DM is characterized by dysregulation of carbohydrate, lipid and protein metabolism, provoked by impaired insulin secretion, insulin resistance, or a combination of both (DeFronzo et al. 2015). Absence of insulin action results in the loss of metabolic control and leads to elevated levels of glucose and lipids in the circulation, increasing the risk for other cardiometabolic conditions. The overt T2DM is preceded by prediabetes, characterized by impaired fasting glucose levels, impaired glucose tolerance, i.e., insulin resistance in muscle or by increased glycated HbA1c levels (DeFronzo et al. 2015). Moreover, it is considered that overnutrition has a fundamental role in T2DM development. Namely, it requires high rates of insulin secretion, which consequently leads to hypersecretion of amylin (a peptide hormone co-secreted with insulin) and to ER stress in β-cells (Poland et al. 2001). Excess of amylin deposits on the β-cell surface, forming amyloid plaques (Matveyenko and Butler 2006), which, in combination with metabolic and ER

stress, elicit β-cell damage, dysfunction and death, causing transition to full-blown T2DM.

Insulin resistance, also denoted as impaired insulin action, is a hallmark of T2DM. The exact aetiology of insulin resistance is still not completely resolved, but is generally thought to stem from obesity-associated exposure of tissues to elevated dietary nutrients, resulting in the accumulation of toxic metabolic by-products (Muoio and Newgard 2008). Furthermore, inflammatory responses and stress related pathways have a considerable role as well, since one of prominent features of insulin resistance is chronic inflammation, characterized by T-cell and macrophage infiltration of visceral adipose tissue (Winer et al. 2011). However, the study by Winer et al. has demonstrated a fundamental pathogenic role for B-cells and IgG in the development of these metabolic abnormalities as well (Winer et al. 2011). Namely, B-cells accumulate in visceral adipose tissue of diet-induced obese mice, however, the mice lacking B-cells are protected from insulin resistance induction despite weight gain. Furthermore, this study showed that infiltrating B lymphocytes exacerbate metabolic disease through production of pathogenic IgG and the high-fat diet (HFD)-related IgG induced insulin resistance through an Fc-mediated process. The crucial distinctive feature of these pathogenic antibodies remained unresolved, despite the fact that the authors examined their antigen-specificity and revealed several autoantibodies directed against a specific profile of self-antigens (Winer et al. 2011). Additional evidence for the involvement of the Fc receptors in insulin resistance induction provided another study, which revealed that inhibitory FcγRIIB is expressed in microvascular endothelium, particularly in the skeletal muscle (Chieko et al. 2005). It was only recently demonstrated that the activation of this particular Fc receptor plays an important role in obesity-induced insulin resistance (Tanigaki et al. 2018). The authors have also shown that the HFD-fed mice lacking B-cells or FcγRIIB (either globally or selectively in endothelium) are protected from obesity-induced insulin resistance, despite an increase in adiposity. Moreover, the same study identified the operating ligand for endothelial FcγRIIB—a distinct hyposialylated IgG glycoform (Fig. 14.2). The observation that hyposialylated IgG is responsible for downstream induction of insulin resistance was confirmed by the transfer of sialidase-treated IgG to B-cell deficient mice on HFD, which resulted in glucose intolerance and insulin resistance (Tanigaki et al. 2018). Hyposialylated IgG has enhanced affinity for endothelial FcγRIIB, whose activation induces insulin resistance due to impaired skeletal muscle glucose uptake, caused by attenuated insulin delivery. This impaired delivery of insulin to skeletal muscle cells is a consequence of decline in insulin transcytosis through endothelium, due to the endothelial nitric oxide synthase antagonism following FcγRIIB activation (Tanigaki et al. 2018; Kubota et al. 2011).

The link between overnutrition, obesity and insulin resistance is quite multidimensional. Obesity has been associated to the increased levels of proinflammatory cytokines, produced by the aforementioned T-cells and macrophages infiltrating visceral adipose tissue. Since adipose tissue expands excessively in obesity and becomes a site of production of multiple inflammation mediators, its pivotal role in the induction of systemic insulin resistance is not surprising (Rosen and Spiegelman 2014). Experimentally, insulin resistance can be induced by incubation of adipocytes with proinflammatory cytokine tumour necrosis factor alpha (TNFα) (Hotamisligil et al. 1993). A study by Parker et al. investigated the effects of an inflammatory insult on protein glycosylation in murine adipocytes upon treatment with TNFα and consequent insulin resistance induction (Parker et al. 2016). The study has demonstrated that insulin resistance affects terminal *N*-glycan epitopes on multiple proteins, as it downregulates α2,3-sialoglycans and upregulates terminal digalactosylated glycans. The observed glycosylation changes coincided with the regulation of the corresponding glycosyltransferases as well. Importantly, insulin resistance induced glycosylation changes on the specific proteins associated with glucose transporter 4 (GLUT4) storage vesicle formation and trafficking (Parker et al. 2016). These proteins function within specialized

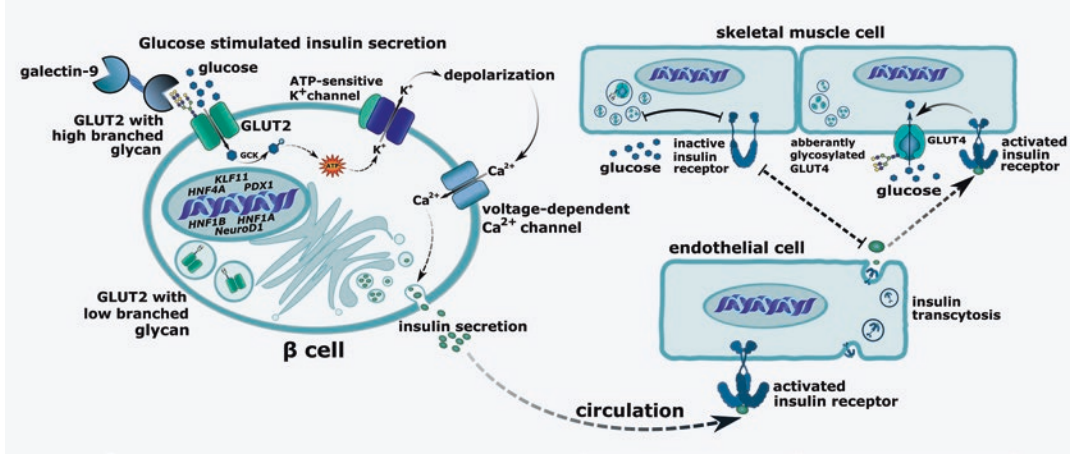


Fig. 14.2 Glycosylation is essential in multiple processes involved in the maintenance of glucose homeostasis. Pancreatic β cell-surface expression of glucose transporter 2 (GLUT2) is essential for glucose-stimulated insulin secretion (GSIS). Glycosyltransferase MGAT4a is responsible for the biosynthesis of high branched *N*-glycans and is required for GLUT2 cell-surface residency. MGAT4a deficiency leads to GLUT2 endocytosis, greatly decreasing cell-surface half-life and impairing GSIS. Proper GLUT2 glycosylation is essential for galectin-9 recognition and sequential lattice formation that enables transporter cell surface membrane retention. GLUT2 enables glucose transport into the β cell, where it is phosphorylated by glucokinase (GCK) and converted into ATP in subsequent metabolic reactions. Increased intracellular levels of ATP alter ion channel activity, eventually leading to the exocytosis of insulin-containing granules, thereby increased insulin levels in circulation. In peripheral tissue insulin stimulates its own transendothelial transport by transcytosis. Insulin binds to the insulin receptor on the endothelial plasma membrane and the complex is transported through the cell. Thereafter, insulin binds to its receptor on the target T-cells and stimulates glucose uptake via glucose transporter 4 (GLUT4). GLUT4 is the insulin-responsive transporter, expressed primarily in muscle and adipose tissue. This transporter bears a single *N*-glycan which plays a critical role in its stability and intracellular trafficking. The absence of glycan residue blunts GLUT4 responsiveness to insulin, by impairing its subcellular localization required for insulin-mediated translocation. Impairment in any of the described processes causes dysregulation of insulin secretion or insulin action, leading to diabetes development

insulin-responsive GLUT4 compartment, essential for coordinated delivery of storage vesicles to the plasma membrane and cell-surface expression of GLUT4 (Olson 2012). Therefore, changes in their glycosylation may modulate their ability to generate GLUT4 storage vesicles, supporting a role for glycosylation in the maintenance of insulin sensitivity.

Fourteen human GLUT proteins are members of the major facilitator superfamily of membrane transporters, responsible for transport of hexoses or polyols (Mueckler and Thorens 2013). One or more GLUT proteins are expressed in virtually every cell of the human body; however, glucose transporters GLUT1-4 are probably the most investigated, with established roles in the control of glucose homeostasis. All GLUT proteins have a single *N*-glycosylation site (Mueckler and Thorens 2013), but the crucial role of this sole *N*-glycan in regulation of the transporter faith has been most extensively studied for GLUT2 and GLUT4. Both glucose transporters play fundamental roles in the maintenance of insulin sensitivity and insulin secretion, whose defects are two cardinal features of T2DM.

Pancreatic β cell-surface expression of GLUT2 is essential for glucose-stimulated insulin secretion (GSIS) and the regulation of glucose homeostasis in response to dietary intake (Ohtsubo et al. 2005). Namely, glucose uptake is the first step in this metabolic-dependent signalling pathway. It has been demonstrated that the murine glycosyltransferase Mgat4a, responsible for the biosynthesis of branched *N*-glycans, is required for Glut2 cell-surface residency (Fig. 14.2) (Ohtsubo et al. 2011). The absence of

Mgat4a leads to Glut2 endocytosis and redistribution to endosomes and lysosomes, greatly decreasing cell-surface half-life and impairing GSIS, which leads to metabolic dysfunction characteristic for T2DM (Ohtsubo et al. 2005). Mgat4a generates third antenna on the bound *N*-glycan, creating an intermediate for the extension with poly-LacNAc, which serves as a ligand for galectin-9 recognition. This recognition enables transporter-galectin lattice formation and sequentially, Glut2 cell surface retention (Ohtsubo et al. 2011). Experiments on human β cells obtained from T2DM donor patients corroborated the aforementioned findings from the mouse models. Namely, these β cells were deficient in MGAT4a expression and GLUT1/GLUT2 cell-surface expression (in normal human islets GLUT1 and GLUT2 are co-expressed), resulting in impaired GSIS as well (Ohtsubo et al. 2013). Herein as well has the absence of branched *N*-glycan induced redistribution of glucose transporter into the lipid-raft microdomain, thereby attenuating cellular glucose uptake. Moreover, it has been shown that the induction of diabetes in mouse model, by chronic ingestion of HFD associates with reduced Mgat4a expression, aberrant Glut2 glycosylation and Glut2 endocytosis (Ohtsubo et al. 2005). Moreover, HFD also reduced the expression of transcription factors FOXA2 and HNF1A, which bind to Mgat4a promoter sequence, consequently leading to its downregulation.

Alongside GLUT2, the crucial regulatory role of a sole *N*-glycan has been demonstrated for GLUT4 as well. Namely, GLUT4 is the insulin-responsive glucose transporter, expressed primarily in muscle and adipose tissue. This transporter also bears a single *N*-glycan which plays a critical role for its stability and intracellular trafficking (Haga et al. 2011). The absence of glycan residue blunts GLUT4 responsiveness to insulin, by impairing its subcellular localization required for insulin-mediated translocation (Fig. 14.2). Aglycosylated GLUT4 is also susceptible to rapid degradation (Ing et al. 1996), however, some studies have indicated that glycosylation has no major role in GLUT4 functional activity, but is rather essential for its efficient trafficking between cellular compartments (Zaarour et al. 2012).

Dietary intake also triggers the release of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) from gut endocrine cells. Binding of the incretins to their dedicated receptors elicits tissue-selective pleiotropic responses, including the increase of GSIS from pancreatic β cells (Whitaker et al. 2012). Both the GIP and GLP-1 receptors possess a large extracellular N-terminus with multiple *N*-glycosylation sites, and it has been observed that *N*-glycosylation promotes cell surface expression of GLP-1R and is required for plasma membrane residency of GIPR (Whitaker et al. 2012). Furthermore, *N*-glycosylation maintains cell surface GIP receptor number and GIP-potentiated insulin secretion, which might have important implications in T2DM aetiology. The reduced incretin effect in human T2DM patients has been observed a long time ago (Nauck et al. 1986), whereas studies in animal models of T2DM demonstrated reduced incretin receptor expression (McIntosh et al. 2009). Thus, future studies would need to determine the exact mechanisms of GIPR and GLP-1R regulation by alternative *N*-glycosylation in T2DM and its implications on incretin response of the β cells. Altogether, these findings are supplying a novel insight into occurring pathophysiological mechanisms during T2DM development, with the crucial participation of aberrant glycosylation.

A fundamental role of overnutrition in T2DM development is also evident on the example of HBP. It is a nutrient-sensing pathway that converts fructose-6-phosphate to UDP-GlcNAc and functions in part as a glucose sensor (Dennis et al. 2009). Hexosamine pathway regulates cellular responses to insulin by controlling the levels of UDP-GlcNAc-mediated glycosylation of targets related to insulin activity (Reily et al. 2019; Traxinger and Marshall 1991). It is also highly responsive to glucose levels, whereas increased hexosamine flux predominantly influences the production of branched, tri- and tetra-antennary *N*-glycans (Lau et al. 2007). Nonetheless, UDP-GlcNAc is not the only product of the pathway, as it serves as a precursor for CMP-Neu5Ac, the

sugar-nucleotide donor utilized by sialyltransferases to cap *N*- and *O*-glycans (Dennis et al. 2009). This would imply that both increased glycan branching and sialylation might direct various processes related to T2DM pathogenesis.

Indeed, several cross-sectional case-control studies have observed increased complexity of plasma protein *N*-glycome in T2DM, that was driven mostly by the increase of highly branched (tri- and tetra-antennary), highly galactosylated and sialylated glycan structures (Keser et al. 2017; Adua et al. 2018; Dotz et al. 2018). These findings are in line with the observations from a GWAS which implicated *MGAT5* gene, encoding the glycosyltransferase responsible for the biosynthesis of tetraantennary glycans, into glucose homeostasis regulation (Bermingham et al. 2018). An identified allelic variant of *MGAT5* gene confers higher T2DM risk and it was associated with enhanced islet expression of the gene. Another study has demonstrated that Mgat5 knockout mice exhibit improved insulin sensitivity (Marshall et al. 1991), however specific effects on the β cells have not been studied. Altogether, this would suggest that the higher expression levels of MGAT5, associated with predisposition for T2DM development, are in accordance with the aforementioned cross-sectional observations, where increased glycan branching was identified in T2DM cases.

Furthermore, quite a few studies investigated IgG glycosylation patterns in T2DM as well (Lemmers et al. 2017; Li et al. 2019; Liu et al. 2019). These cross-sectional studies have demonstrated that similar IgG glycosylation alterations occur in T2DM in various independent populations. In brief, T2DM-related changes included decreased galactosylation and sialyation, an increase in fucosylated structures with bisecting GlcNAc and a decrease in fucosylated structures without bisecting GlcNAc, indicating an enhanced pro-inflammatory potential of the circulating IgG.

Altogether, it would seem that T2DM associates with variable alterations in protein sialylation, depending on the cell of origin of the studied protein. This notion is further supported by a finding of the inverse regulation of α2,3- and α2,6-sialylation of plasma proteins in T2DM (Dotz et al. 2018). Specifically, α2,3-sialylation was decreased in diabetes cases, which coincides with previous reports on downregulation of α2,3-sialyltransferease activity by proinflammatory cytokines (TNFα) during induction of insulin resistance (Parker et al. 2016). Oppose to decreased α2,3-sialylation, increase in α2,6-sialylation of biantennary glycans (which are usually found on IgG) was observed (Dotz et al. 2018), which coincides with the previous reports on T2DM-related changes of IgG glycosylation (Lemmers et al. 2017; Li et al. 2019). The notion of tissue-specific regulation of protein sialylation in diabetes is further supported by the findings from a recent large GWAS, which investigated genetic loci implicated in T2DM predisposition, and included genetic data from nearly 900,000 individuals (Mahajan et al. 2018). This GWAS resulted in identification of a T2DM risk allele variant in *ST6GAL1* gene, encoding α2,6-sialyltransferase. This T2DM risk conferring allelic variant associates with increased, islet-specific ST6GAL1 expression, that has been previously linked to decreased insulin secretion during provocative testing in the variant-carriers (Mahajan et al. 2018). Increased sialylation in pancreatic β cells might lead to the formation of terminal sialic-acid linkages on the GLUT2 *N*-glycan, which would then likely mask the lectin ligand and thereby attenuate cell-surface GLUT2 expression (Ohtsubo et al. 2005), as already discussed. Nonetheless, different effects were observed in mouse adipose tissue, as *St6gal1* knockout mice had increased body weight and accumulation of visceral fat, implicating a role for St6gal1 in adipogenesis (Kaburagi et al. 2017). These findings were not supported by human GWAS studies, as *ST6GAL1* variants did not show significant associations with body fat distribution traits nor with body mass index (Shungin et al. 2015; Locke et al. 2015). Altogether, these findings are implying that the effects of *ST6GAL1* variants are likely mediated through certain tissue-specific regulatory mechanisms.

Accumulating evidences are clearly demonstrating that *N*-glycosylation exerts important

functions related to glucose sensing, glucose cellular uptake, insulin secretion and sensitivity. Defect in any of these processes impairs glucose homeostasis and leads to onset of diabetes. Since glycans have shown to be very responsive to various pathophysiological conditions, an assumption that their changes could reflect underlying processes preceding T2DM onset was legitimate. Couple of studies explored the prospect of using glycosylation alterations on plasma glycoproteins as molecular risk markers and prediction tools (Keser et al. 2017; Wittenbecher et al. 2020). Namely, these studies attempted to distinguish individuals at an increased risk of T2DM development among baseline-healthy study participants, based on interindividual differences in plasma protein glycosylation. The first study has demonstrated that the individuals with increased T2DM risk, incident cases of T2DM collected at baseline, and individuals with elevated HbA1c have all presented with the increased plasma protein glycan branching, galactosylation and sialylation (Keser et al. 2017). Sequential prospective follow-up of the EPIC-Potsdam cohort confirmed that changes in plasma *N*-glycome composition are predictive of incident T2DM (Wittenbecher et al. 2020). Namely, plasma protein glycome had the ability to identify at-risk individuals around 6.5 years prior to the diabetes onset, with rather successful predictive performance of AUC of 0.83. Additionally, the information conferred by selected predictive glycans improved the accuracy of the established and clinically applied German Diabetes Risk Score (Paprott et al. 2016). These data are indicating that plasma glycome profiling represents a promising tool in T2DM risk stratification approaches.

Cardiometabolic diseases, including T2DM represent the leading cause of disability and death in the world. Diabetes is a serious illness that diminishes quality of life due to its specific complications. Namely, individuals with T2DM are at high risk for both microvascular complications (such as retinopathy, nephropathy and neuropathy) and macrovascular complications (such as cardiovascular comorbidities), provoked by hyperglycaemia and insulin resistance (metabolic) syndrome (DeFronzo et al. 2015). Several epidemiological and prospective studies suggest that an early intensive control of hyperglycaemia is able to decrease the risk of micro- and macrovascular complications, however, their occurrence still remains an unsolved issue (Testa et al. 2017). Therefore, the utility of glycan markers has been examined in the context of diabetic complications as well. Specifically, associations of both plasma and IgG *N*-glycome alterations with the course of kidney function in T2DM have been investigated (Singh et al. 2020; Adua et al. 2018). Chronic kidney disease (CKD) is one of the most frequent diabetic complications, as nearly half of all T2DM patients may suffer from kidney dysfunction at some stage in their life (Câmara et al. 2017). CKD is diagnosed based on kidney damage or decline in renal function over a 3-month period, estimated by eGFR. Therefore, glycomic studies mostly investigated the link between glycosylation features and eGFR. Namely, it has been observed that fucosylated IgG glycoforms bearing bisecting GlcNAc significantly associate with a faster decline in eGFR (Singh et al. 2020). Conversely, fucosylation of neutral and monogalactosylated structures was associated with lowered eGFR annual decline. Altogether, these IgG *N*-glycosylation patterns, reflecting a pro-inflammatory state of circulating IgG at baseline, associated prospectively with a faster decline of kidney function in patients with T2DM. Furthermore, monitoring of the plasma glycome alterations revealed that T2DM patients with concomitant CKD show decrease in biantennary galactosylated and sialylated glycans compared to diabetics without CKD (Adua et al. 2018). Naturally, the potential of glycans as diabetes complication markers warrants further research. Nonetheless, any biomarker that would provide additional information, alongside known risk factors, could aid in better prediction and tailored treatment to prevent and delay kidney function decline.

14.6 Gestational Diabetes

Gestational diabetes could be, in simplest terms, defined as hyperglycaemia that develops during second or third trimester of pregnancy, which was not clearly overt diabetes prior to gestation and which resolves after childbirth (McIntyre et al. 2019). Gestational diabetes is currently the most common medical complication of pregnancy, conferring increased cardiometabolic risks for both mother and the foetus. Moreover, diagnosis of gestational diabetes identifies women and their infants who are at higher risk of developing diabetes, obesity and premature cardiovascular disease in the future (McIntyre et al. 2019; Daly et al. 2018). Due to lack of the uniform worldwide consensus on diagnostic criteria for gestational diabetes, it is hard to accurately estimate its prevalence, but available reports are suggesting that gestational diabetes occurs in 1% to >30% of pregnancies (Zhu and Zhang 2016). Generally, pregnancy represents a sort of metabolic stress test, indicative of future metabolic disorders. Namely, normal pregnancy encompasses extensive metabolic alterations, including a 30% increase in basal endogenous glucose production, increase in fasting insulin levels, decrease in circulating fasting glucose levels due to increased plasma volume and glucose utilization by foetus, a 50% decrease in peripheral insulin sensitivity, and finally, a 2–3-fold increase in insulin secretion in response to the decreased insulin sensitivity which preserves normoglycaemia (McIntyre et al. 2019). It is not unusual for the women who are initially healthy, but develop gestational diabetes during pregnancy, to have a decreased peripheral insulin sensitivity or β-cell dysfunction even before conception (Catalano et al. 1999). At the beginning of gestation, these women remain normoglycaemic due to increased β-cell insulin response, however, as the insulin resistance increases with the advancing pregnancy, the insulin response becomes inadequate, which results in hyperglycaemia (McIntyre et al. 2019). To date, attempts at prevention of gestational diabetes through lifestyle, dietary, or drug interventions, have not shown consistent benefits and none could be recommended for routine use (McIntyre et al. 2019), making optimal long-term management of mother and infant challenging.

In the context of glycomic research in gestational diabetes, there are only a few studies that tackled the field, most of them focusing on secretory glycoproteins. One study investigated gestational diabetes-related alterations in glycosylation of glycodelin-A, an abundant decidual glycoprotein with glycosylation-dependent immunomodulatory activities (Lee et al. 2011). Glycodelin-A is implicated in fetomaternal defence through these immunomodulatory activities, including induction of T-cell apoptosis and modulation of natural killer, B and dendritic cell activities. The study investigated whether aberrant carbohydrate metabolism in gestational diabetes associates with changes in glycodelin-A glycosylation, causing defective immunomodulatory activities (Lee et al. 2011). It was revealed that gestational diabetes induced a decrease in α2,6-sialylated and high-mannose glycans and an increase in glycan structures with Sda epitopes (NeuAcα2,3[GalNAcβ1,4]Gal). The observed decrease in α2,6-sialylation might be explained by increased placental sialidase activity in gestational diabetes. Complete removal of sialic acids abolished the immunomodulation and binding of glycodelin-A. Moreover, glycodelin-A from gestational diabetes cases exhibited reduced immunosuppressive activities in terms of cytotoxicity on lymphocytes, inhibitory activities on IL-2 secretion, stimulatory activities on IL-6 secretion by NK cells, and binding to these cells (Lee et al. 2011). These findings are indicating that altered glycosylation of glycodelin-A in gestational diabetes impairs its bioactivity.

Furthermore, diabetic pregnancy has been associated with glycosylation changes of human chorionic gonadotrophin (hCG), a glycoprotein hormone that is produced by normal trophoblasT-cells of the placenta during pregnancy (Elliott et al. 2007). During the early pregnancy, the secretion of hCG stimulates ovarian production of progesterone, essential for successful zygote implantation and maintenance of pregnancy. Moreover, it appears that hCG also exerts certain immuno- and invasion-suppressive activities. This study revealed that both α and β subunits of

diabetic hCG predominantly bear unusual biantennary *N*-glycan (both GlcNAc antennae attached to α1,3-mannose arm), that was previously reported only in carcinoma (Elliott et al. 2007). Moreover, fucosylated triantennary hCG glycans were substantially increased, whereas hCG sialylation was notably reduced in diabetic pregnancy, however the functional implications of these changes have yet to be elucidated.

Placental transferrin receptor is another glycoprotein whose glycosylation alters in gestational diabetes (Georgieff et al. 1997). This receptor is needed for adequate maternal-foetal iron transport, which is usually hindered in gestational diabetes, resulting in foetal iron deficiency. Despite increased expression of the receptor, binding to diferric transferrin is decreased proportionately to the severity of maternal diabetes (Georgieff et al. 1997). The study revealed that transferrin receptors isolated from diabetic placentae have increased glycosylation, which potentially affects the three-dimensional structure or charge of the receptor, leading to altered binding kinetics to diferric transferrin.

It was also observed that gestational diabetes impacts the *N*-glycosylation of milk immunemodulatory proteins, that are implicated in modulation of infant immunity (Smilowitz et al. 2013). Glycosylation and concentration alterations were observed for both secretory immunoglobulin A (sIgA) and lactoferrin. The gestational diabetes associated with substantailly lower sIgA concentration, and with decrease in sIgA mannose, fucose and sialic acid *N*-glycans. Conversely, the concentration of lactoferrin increased in gestational diabetes, and the abundance of lactoferrin fucose and sialic acid *N*-glycans were also increased (Smilowitz et al. 2013). This protein-dependent glycosylation changes imply differential dysregulation in plasma B-cells and mammary epithelial cells, as a consequence of gestational diabetes-related hyperglycaemia, oxidative stress and hyperinsulinemia.

All of these studies are demonstrating that glycosylation alterations in gestational diabetes might have lasting implications for both maternal and infant immune function and intestinal microbiome. Therefore, approaches aimed at fixing the glycosylation changes, or at the development of specific nutritional care for the affected infants, may help to alleviate some of the complications associated with gestational diabetes.

14.7 Monogenic Diabetes

The field of glycosylation alterations and their implications in the group of monogenic and rare diabetes is still quite underexplored. Part of the reason lies in the fact that monogenic diabetes accounts for very small percentage of the total diabetes cases, in addition to the great heterogeneity between different subtypes. In contrast to the common diabetes types that are complex polygenic disorders, monogenic diabetes encompasses a group of single gene disorders, commonly characterized by functional defects of pancreatic β cells that cause moderate to severe hyperglycaemia (Flannick et al. 2016). Maturity onset diabetes of the young (MODY) is a subgroup of diverse monogenic autosomal dominant disorders, caused by variants in one of the 13 different genes (Ellard et al. 2013). The most frequent adult form is caused by variants in the *HNF1A* (hepatocyte nuclear factor 1-alpha) gene and is denoted as HNF1A-MODY. The *HNF1A* gene is expressed in many cell types and tissues, including pancreatic islets, liver, kidney and intestine, but not in skeletal muscle or visceral adipose tissue (Harries et al. 2006). The gene encodes a transcription factor HNF1A, which regulates the expression of numerous β-cell genes, including genes for insulin, pyruvate kinase and GLUT2 (Harries et al. 2006). The *HNF1A* mutations show a great penetrance, evident through progressive deterioration of β-cell function, due to their inability to increase insulin secretion in response to elevated glucose levels. It is estimated that up to 80% of HNF1A-MODY cases remain undiagnosed or misdiagnosed as T1DM or T2DM (Pavić et al. 2018; Shields et al. 2010), creating the pressing need for reliable clinical markers and diagnostic algorithms. Moreover, identification of a rare *HNF1A* variant by sequencing still does not enable a definite

diagnosis of HNF1A-MODY, since it has been demonstrated by large-scale sequencing projects that many gene variants are present in healthy people as well (Flannick et al. 2013). Therefore, a functional interpretation and systematic assessment of disease causality is required for previously non-reported mutations.

The potential of glycan biomarkers in advancing the field of MODY diagnostics has been unravelled by the first glycan-based GWAS study (Lauc et al. 2010), which identified HNF1A as a master regulator of plasma protein fucosylation. This link between HNF1A and glycosylation was not previously observed. HNF1A was shown to regulate the expression of several genes involved in the protein fucosylation. Namely, HNF1A regulates the expression of genes encoding enzymes involved in de novo and salvage biosynthetic pathways (i.e., GDP-mannose-4,6-dehydratase and fucokinase, respectively), where GDP-L-fucose, a substrate for both core and antennary fucosyltransferases, is produced (Lauc et al. 2010). Additionally, HNF1A directly regulates the expression of several fucosyltransferase (*FUT*) genes. Specifically, it upregulates antennary fucosyltransferases (FUT3, FUT5 and FUT6) and downregulates core fucosyltransferase (FUT8), thereby decreasing the consumption of GDP-L-fucose by FUT8 and additionally increasing the fucose bioavailability to antennary fucosyltransferases. The fact that HNF1A 'promotes' antennary fucosylation has been utilized in the sequential study, which has demonstrated that MODY-causing *HNF1A* mutations associate with marked alterations in plasma protein fucosylation (Thanabalasingham et al. 2013). Specifically, HNF1A-MODY was associated with substantial decrease in antennary fucosylated glycans, therefore, a plasma fucosylation index that summarizes the proportion of antennary fucosylated triantennary glycans has been built (Thanabalasingham et al. 2013). This fucosylation index was highly successful in discrimination of HNF1A-MODY from other types of diabetes (type 1, type 2 and glucokinase-MODY), which made it a promising diagnostic marker. Nonetheless, the study had certain limitations, as it used groups of clinically well-defined patients with confident diagnosis based on clinical, biochemical and molecular investigations, which did not reflect a real-life clinical setting. Therefore, a sequential study was conducted, which recruited an unselected population of patients with a diagnosis of young adult-onset non-autoimmune diabetes (Johannes et al. 2018), in order to assess the true clinical value of glycan-based markers for HNF1A-MODY. This follow-up study has confirmed the great clinical potential of plasma antennary fucosylated glycans, as it was demonstrated that they have the ability to successfully differentiate diabetic subjects with damaging *HNF1A* alleles from those without rare *HNF1A* alleles. Specifically, the most informative triantennary, disialylated, antennary fucosylated glycan structure (A3F1G3S2) showed excellent classification performance with AUC of 0.90, corresponding to 88% sensitivity and 80% specificity. Furthermore, fucosylated glycans aided in assigning a disease causality of the identified rare *HNF1A* alleles. Namely, subjects harbouring likely damaging *HNF1A* alleles consistently exhibited significantly lower levels of antennary fucosylated glycans than individuals with likely benign *HNF1A* alleles. Moreover, fucosylated glycans also demonstrated consistency in assigning a direction of the functional effect in individuals harbouring *HNF1A* variants of unknown significance. All of these findings are showing that antennary fucosylated plasma glycans have promising future prospects as a part of diagnostic algorithms that would combine clinical features with glycan biomarkers. In case their performance as diagnostic markers would not suffice, they could at least improve the selection of subjects referred to *HNF1A* sequencing.

Finally, in addition to the aforementioned β-cell genes and fucosylation-related genes, HNF1A likely regulates the expression of another glycosyltransferase—MGAT4. Namely, *MGAT4* gene contains promoter sequence with binding sites for HNF1A (Ohtsubo et al. 2005). Considering the importance of MGAT4-constructed branched glycan for GLUT2 cell surface retention, it would not be surprising that functional mutations in *HNF1A* also impair GLUT2 trafficking, further contributing to the β-cell dysfunction and diabetes pathogenesis.

Alongside HNF1A-MODY, the glycosylation changes have been linked to one more subtype—CEL-MODY. This subtype belongs to the group of syndromic diabetes, in which features other than diabetes itself dominate the clinical phenotype, as exocrine pancreas dysfunction characterizes CEL–MODY (Flannick et al. 2016). CEL-MODY is caused by nonsense point mutations in *CEL* gene, which is mainly expressed in mammary glands and pancreatic acinar tissue, demonstrating that T-cell types other than the β-cell might be implicated in diabetes (Anık et al. 2015). The *CEL* gene encodes enzyme carboxyl ester lipase, a bile-salt-dependent/responsive lipase that is activated after secretion into the intestines, which participates in the hydrolysis and absorption of cholesterol and fat-soluble vitamins. CEL-MODY is characterized by exocrine pancreatic dysfunction during the childhood and diabetes in adulthood, however, the exact pathogenesis of pancreatic lipomatosis and exocrine pancreatic dysfunction still remains unknown (Anık et al. 2015). Some reports are indicating that differentially *O*-glycosylated carboxyl ester lipase could negatively affect β-cell function (Wolters-Eisfeld et al. 2018). Namely, it was demonstrated that the impairment of glycosyltransferase core 1 synthase, leads to aberrant *O*-glycosylation (overexpression of truncated *O*-glycans, i.e., Tn antigen), which results in exocrine pancreatic insufficiency with decreased activities of digestive enzymes and diabetes. Carboxyl ester lipase is heavily *O*-glycosylated at the protein C-terminus and *O*-glycosylation plays a role in proper folding, secretion and stability of the enzyme (Bruneau et al. 1997). Moreover, the loss of CEL core 1-derived *O*-glycans has effects analogous to frameshift mutations in the *CEL* gene. Therefore, these findings are demonstrating the importance of correct *O*-glycosylation for normal exocrine and endocrine function of pancreas. The CEL enzyme also bears a single site for *N*-glycosylation, however, its potential alterations and their involvement in initiation/progression of the CEL-MODY, remain to be investigated.

14.8 Perspective

In this chapter we attempted to provide a summarized overview of the most prominent findings involving changes of protein *N*-glycosylation in various diabetes subtypes. A growing body of evidence is demonstrating that *N*-glycosylation is essential in multiple processes involved in the maintenance of glucose homeostasis. Moreover, specific protein glycoforms and changes in *N*-glycan composition have been identified in nearly all diabetes subtypes. Previous, current and future research has been and will be focusing on search for markers that could identify predisposition for diabetes development, differentiate between the various subtypes, establish early diagnosis, predict complications and represent novel therapeutic targets. In this context, *N*-glycans seem to have a very promising potential, however, substantial research efforts still need to be invested to achieve that goal.

Compliance with Ethical Standards

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Glycosylation and Cardiovascular Diseases

15

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Abstract

Cardiovascular disease (CVD) is the leading cause of death worldwide, accounting for approximately 18 million deaths in 2017. Coronary artery disease is the predominant cause of death from CVD, followed by stroke. Owing to recent technological advancements, glycans and glycosylation patterns of proteins have been investigated in association with CVD risk factors and clinical events. These studies have found significant associations of glycans as biomarkers of systemic inflammation and major CVD risk factors and events. While more limited, studies have also shown that glycans may be useful for monitoring response to anti-inflammatory therapies and may be responsive to changes in lifestyle, particularly in patients with chronic inflammatory diseases. Glycans capture summative risk information related to inflammatory, immune, and signaling pathways and are promising biomarkers for CVD risk prediction and therapeutic monitoring.

Keywords

Cardiovascular disease · Glycosylation · Inflammation · Immunoglobulin G · Prevention · Biomarkers

15.1 Introduction

Globally, cardiovascular disease (CVD) death rates vary by country (highest in Eastern Europe and Central Asia) and within countries. During the past decade, total CVD deaths have increased globally by approximately 20%, and at the same time, the age-adjusted cardiovascular deaths have decreased by approximately 10% (Virani et al. 2020). CVD still accounts for >2000 deaths each day in the United States (Benjamin et al. 2019). Despite the general decline in age-adjusted CVD death rates, the total number of CVD deaths has increased due to both population growth and aging and in some countries due to increases in

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age-specific cardiovascular mortality rates which may be related to rising obesity and diabetes rates (Virani et al. 2020). Recent global increases in obesity and diabetes and their long-term consequences on cardiovascular mortality are the biggest current challenges in cardiovascular prevention and may have a significant negative impact on cardiovascular mortality rates, as noted in the recent blunting in the decline of CVD death rates. The decline in age-adjusted CVD mortality that has been seen over the past several decades may be ending, and new approaches will be required to change this trajectory (Roth et al. 2018). Experimental and observational studies on prevention and treatment of CVD have shown the fundamental role of chronic inflammation (Moore 2019) and dysregulation of the immune system (Fernández-Ruiz 2016; Swirski and Nahrendorf 2018) in the pathogenesis of atherosclerotic CVD (Libby et al. 2011; Ross 1999; Hansson 2005; Lawler and Mora 2016). Inflammation characterizes all phases of CVD development and provides a critical pathophysiologic link between atherosclerotic plaque formation and acute plaque rupture, leading to occlusion, infarction, and clinical events (e.g., myocardial infarction, stroke) (Ridker 2020). During chronic inflammation, inflammatory proteins not only experience changes in circulatory concentrations but also in their glycan structures, leading to modifications in the interaction with receptors and changes in their function (Arnold et al. 2008; Gornik and Lauc 2008; Gornik et al. 2012; Lauc et al. 2016). The different types of oligosaccharide structures, sequences, and linkage present in glycoproteins make the human glycome widely diverse (Hart and Copeland 2010; Davids et al. 2019; Suhre et al. 2019). These structural diversities allow glycans to be involved in a wide range of functions (e.g., hormone, ion-channel, and immune regulation) and glycans serve in various essential cellular mechanisms and molecular pathways, including acute phase and chronic inflammatory pathways (Sukhorukov et al. 2019; Varki 2017). Because of the key involvement of glycans and glycoproteins in systemic inflammation, glycans provide a novel perspective for understanding the molecular mechanisms that lead to the development of atherosclerosis and clinical CVD events and have the potential to translate into targets for new therapies and biomarker discovery (Stewart et al. 2017; Nagai-Okatani and Minamino 2016; Magadum et al. 2018).

15.2 Laboratory Measurement

Given their diversity, measuring glycans was previously difficult because of the lack of sensitive, rapid, and high-throughput technology (Yang et al. 2017; Etxebarria and Reichardt 2016; Otvos et al. 2015; Alley et al. 2013). Since the late 1980s, nuclear magnetic resonance (NMR) spectroscopy, in particular one-dimensional proton 1D-^{1}H NMR, was used to identify fingerprints of glycoproteins in blood plasma (Bell et al. 1987). Recently, a reference laboratory developed an algorithm to quantify the prominent resonance of plasma detected by NMR originating from the *N*-acetyl methyl group protons of mobile glycan residues from glycosylated acute phase reactant proteins: mainly, α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin, and transferrin (Otvos et al. 2015). This signal was named GlycA (Otvos et al. 2015). Other laboratories have also developed methods to quantify glycoproteins and GlycA in serum samples based on NMR (Fuertes-Martín et al. 2020; Ellul et al. 2019). These methods employ different proprietary extraction algorithms for the NMR signal (e.g., Bayesian regression models versus line shape deconvolution), and direct comparisons across laboratories and methods are limited (Remaley and Otvos 2020).

In addition, mass spectrometry (MS) and hydrophilic-interaction ultra-high-performance liquid chromatography with fluorescence detection (HILIC-UHPLC-FLD) techniques have been utilized to identify a wide range of *N*- and *O*-linked glycans in biological samples. There have been substantial international efforts to build glycans structural libraries (Aoki-Kinoshita et al. 2016) and to address reproducibility and standardization issues of these techniques (Cummings and Pierce 2014; Parodi et al. 2017).

As a result, relative concentrations of a wide range of immunoglobulin G (IgG) and other plasma glycans can now be measured (Clerc et al. 2018; Trbojević-Akmačić et al. 2016).

15.3 Glycans as Biomarkers for Cardiovascular Disease

Posttranslational modifications of proteins via enzymatic regulation of glycans have been explored in order to identify new biomarkers of CVD development and progression (Gudelj and Lauc 2018; Tibuakuu et al. 2019; Menni et al. 2018; Ritchie et al. 2015). GlycA as well as IgG and plasma protein glycan signatures have shown promising results. GlycA is a composite biomarker and, as such, may be more sensitive to detect systematic inflammatory states compared to traditional inflammatory biomarkers such as high-sensitivity C-reactive protein (hsCRP) (Otvos et al. 2015; Gruppen et al. 2015). For example, hsCRP is a widely used inflammatory clinical marker and part of the early acute phase response, whereas GlycA is a reflection of a complex acute phase response (Muhlestein et al. 2018), possibly having less variability than other inflammatory proteins classically used. Moreover, IgG glycan signatures can be used as an excellent marker of the general health state of individuals (de Jong et al. 2016; Gudelj et al. 2018). Studies have shown that IgG galactosylation and sialylation have been strongly associated with hsCRP levels and poor metabolic health in both male and female individuals (Gudelj et al. 2018; Plomp et al. 2017). Signatures of IgG glycans have also been associated with systemic immune responses and cardiovascular health (Gudelj and Lauc 2018; van den Hoogen et al. 2019).

15.3.1 Primary Prevention of CVD

Primary prevention of CVD aims to prevent the clinical manifestation of CVD in individuals without manifest CVD, through lifestyle approaches and treatment of CVD risk factors, in particular among high-risk individuals (Gupta and Wood 2019). Studies have shown that glycosylation disorders and alterations in *N*-glycosylation may be associated with known major CVD risk factors (Wang et al. 2019; Knežević et al. 2010). The major CVD risk factors are included in the sex- and race-adjusted CVD risk prediction models (Goff et al. 2014) for calculating a 10-year risk of CVD events that is recommended by the American Heart Association and the American College of Cardiology for risk stratification and screening of all asymptomatic American adults age 40–75 years old (Arnett et al. 2019). Likewise, these major CVD risk factors are also included in the European SCORE (Systematic Coronary Risk Evaluation, predicting fatal cardiovascular events) (Conroy et al. 2003) and the World Health Organization CVD risk charts for 21 world regions (Group 2019; Mach et al. 2019). A summary of observational studies examining glycosylation markers with CVD risk factors is shown in Table 15.1.

There is also evidence that increased body fat is associated with increased non-galactosylated *N*-glycans and sialylation of biantennary structures, in addition to decreased digalactosylated *N*-glycans (Knežević et al. 2010). It has been shown that GlycA is positively associated with dysfunctional adipose tissue, leptin resistance, and insulin sensitivity index (Dullaart et al. 2015; Lorenzo et al. 2017). Patients with more favorable cardiovascular health metrics have lower GlycA levels (Benson et al. 2018), and elevated levels of GlycA and IgG FA2G2S1 have been associated with increased risk of developing type 2 diabetes and CVD events (Menni et al. 2018; Akinkuolie et al. 2015; Connelly et al. 2016). Thus, GlycA levels and glycosylation patterns may indicate the chronic low-grade inflammatory state characteristic of patients with obesity and metabolic syndrome (Chandler et al. 2016; Manmadhan et al. 2017, 2019; Mokkala et al. 2020), making glycans promising markers to improve risk stratification for the primary prevention of CVD.

Akinkuolie et al. (2014) examined 28,000 samples from the Women's Health Study that enrolled middle-aged and older healthy American female healthcare workers who were

Table 15.1 Summary of epidemiological studies examining glycosylation markers and cardiovascular disease (CVD) risk factors and outcomes

CVD risk factors	Summary studies
Age	Pro-inflammatory *N*-glycosylation of IgG traits increase with age (Gudelj and Lauc 2018; Menni et al. 2018; Catera et al. 2016; Ruhaak et al. 2010)
Sex	Men and postmenopausal women have decreased levels of IgG galactosylation compared to premenopausal women (Chen et al. 2012; Ercan et al. 2017; Krištić et al. 2014)
Ethnicity	Different patterns of IgG glycosylation are seen in European, African, and South American populations, although their relationship with CVD is yet to be defined (de Jong et al. 2016)
HDL and LDL	Elevated levels of GlycA are related to impaired HDL function and metabolism (Riggs et al. 2019). The HDL glycoprotein composition is correlated to its immunomodulatory capacity (Krishnan et al. 2017; Kailemia et al. 2018; van den Boogert Marjolein et al. 2019; Liu et al. 2018a). Lower sialylation of LDL is related to atherogenesis (Orekhov et al. 2014) and higher sialylation is seen in patients receiving statins (Liu et al. 2018a; Lindbohm et al. 2000). Higher total cholesterol and non-HDL cholesterol are positively associated with GlycA levels (Gruppen et al. 2015, 2019a; Akinkuolie et al. 2014; Dullaart et al. 2015)
Triglycerides	Concentrations of both GlycA (Connelly et al. 2017) and IgG glycans (Liu et al. 2018a) have been shown to be strongly correlated with levels of triglycerides
Systolic blood pressure	Certain patterns of IgG glycosylation (decreased levels of galactosylation, sialylation, and fucosylation) are related to hypertension and prehypertension (Liu et al. 2018a; Wang et al. 2016; Gao et al. 2017). Subjects with elevated GlycA levels are more likely to have hypertension (Gruppen et al. 2019b)
Smoking status	Acute and chronic exposure to cigarette smoke is related to elevated levels of GlycA (Kianoush et al. 2017). Fucosylated surfactant protein-D has been related to COPD development in smokers (Ito et al. 2015). The increase of certain IgG glycosylation traits have been observed in smokers (Wahl et al. 2018)
Type 2 diabetes mellitus	High GlycA levels predict the future risk of type 2 diabetes (Gruppen et al. 2019a; Akinkuolie Akintunde et al. 2015). Certain IgG *N*-glycans (e.g., FA2B, FA2[6]G1, FA2[3]BG1) and traits are associated with type 2 diabetes (Wittenbecher et al. 2020; Lemmers et al. 2017)
Inflammation	GlycA levels (Otvos et al. 2015; Lawler et al. 2016; Chandler et al. 2016) and IgG *N*-glycan signatures (e.g., IgG1, bisecting GlcNAc, sialylation and core fucosylation) (de Jong et al. 2016; Gudelj et al. 2018; Šimurina et al. 2018) are indicators of systemic inflammation
CVD events	Glycans are associated with the risk of primary and secondary prevention of CVD (Gudelj and Lauc 2018). Elevated GlycA levels (Gruppen et al. 2015; Akinkuolie et al. 2014, 2016; Lawler et al. 2016; Duprez et al. 2016) and IgG *N*- and *O*-glycans (Magadum et al. 2018; Menni et al. 2018; Che et al. 2020; Cubedo et al. 2014; Liu et al. 2018b; Heijmans et al. 2019) are associated with myocardial infarction, ischemic stroke, hospitalization for stable angina, coronary revascularization, heart failure, and CVD death. Subclinical CAD (including in patients with chronic inflammatory conditions), PAD, presence of femoral and carotid plaques, as well as development of abdominal aortic aneurysm and extremity thrombosis are associated with GlycA levels (Menni et al. 2018; Joshi et al. 2016; Ormseth et al. 2015; Purmalek et al. 2019; Fashanu et al. 2019) and IgG glycans (Menni et al. 2018; Wang et al. 2019; Varki et al. 2008; Li et al. 2018; Jandus et al. 2019)

IgG immunoglobulin G, *CVD* cardiovascular disease, *GlycA* glycoprotein acetylation, *HDL* high-density lipoproteins, *LDL* low-density lipoproteins, *COPD* chronic obstructive pulmonary disease, *CAD* coronary artery disease, *PAD* peripheral artery disease

followed for a median of 17 years. Elevated baseline concentrations of GlycA were associated with a greater risk of incident CVD events independent of major CVD risk factors (Akinkuolie et al. 2014). These results have been subsequently validated in different groups, including patients of both genders and different ethnicities that have participated in some of the biggest CVD primary prevention studies such as the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (Akinkuolie et al. 2016), Multi-Ethnic Study of Atherosclerosis (Duprez et al. 2016), and Prevention of Renal and Vascular End stage Disease (Gruppen et al. 2015), among others (Harada et al. 2019; Kettunen et al. 2018). Additionally, elevated GlycA levels were also associated with the development of heart failure (Kettunen et al. 2018) and cardiovascular mortality (Lawler et al. 2016).

GlycA has also been linked to the presence of subclinical noncoronary atherosclerosis, more specifically to the presence of femoral and carotid plaques (Menni et al. 2018; Fashanu et al. 2019), peripheral artery disease (PAD) (Fashanu et al. 2019), as well as extra-coronary calcifications in the aortic valve, mitral annulus, and ascending and descending thoracic aorta (Ezeigwe et al. 2019). Not only were elevated levels of GlycA related to asymptomatic disease, but they were also associated with 2-year progression of the mitral annulus and descending thoracic aorta calcifications (Ezeigwe et al. 2019) as well as increased risk for development of clinical PAD events, such as lower extremity claudication, abdominal aneurysm, arterial embolism, and/or thrombosis of the lower extremity after years of follow-up (Fashanu et al. 2019).

The association between GlycA and CVD is of a magnitude comparable to hsCRP, an inflammatory biomarker that has been validated and used for cardiovascular risk stratification for many years (Ridker et al. 2000; Ridker 2001). Interestingly in some studies, the association of GlycA to CVD development was weaker after 6 years of follow-up (Akinkuolie et al. 2014). Glycan conjugates undergo structural changes based on the phenotypic and metabolic state of a cell, making them very dynamic markers (Hart and Copeland 2010; Dashti et al. 2020), as such glycans may be good biomarkers for short- and medium-term development of CV events compared to other inflammatory biomarkers such as hsCRP (Akinkuolie et al. 2014).

Glycosylation studies investigated molecular mechanisms of CVD events that provide evidence for linking IgG glycans to cardiometabolic risk and subclinical atherosclerosis (Menni et al. 2018; Wittenbecher et al. 2020; Gudelj and Lauc 2018; Tijardović et al. 2019; Heijmans et al. 2019; Liu et al. 2018a; Russell et al. 2019). A study on 2970 women from the TwinsUK and its replication on 967 women from the Orkney Complex Disease Study (Menni et al. 2018) identified a significant association between 10 glycan traits and the 10-year risk of CVD events. From these, 6 glycans were significantly associated with CVD risk in men (N = 845). The authors take advantage of these significant associations to introduce a glycan-based CVD risk score using IgG glycans (FA2G2, FA2G2S1, FA2B/GPn, FA2[3]G1/GPn, where GPn are total neutral IgG glycans) and GlycA, which strongly correlated with the 10-year risk score ($P = 2.1x10^{-110}$). Significant associations between these glycans and subclinical noncoronary atherosclerosis (femoral and carotid plaque) have been also reported. The recent study (Wittenbecher et al. 2020) from the European Prospective Investigation of Cancer (EPIC) which was replicated on the Finland Cardiovascular Risk Study (FINRISK) showed predictive values of several total plasma *N*-glycans, including FA2G2S1 and A2G2S2, for calculating the risk of type 2 diabetes and CVD events in these cohorts.

15.3.2 Patients with Inflammatory Diseases

Patients with chronic inflammatory conditions like autoimmune diseases or human immunodeficiency virus (HIV) are at increased risk for CVD.

In this population traditional nonspecific biomarkers such as hsCRP may not be adequate predictors for CV events (Joshi et al. 2016; Borges et al. 2016). Recent studies have identified GlycA as a CVD biomarker for these patients. Signatures of IgG glycans have been shown to be associated to the severity of inflammatory diseases (Plomp et al. 2017; Dekkers et al. 2018; Scheepers et al. 2017) and also manifestations of infectious diseases such as HIV (Ackerman et al. 2013; Vadrevu et al. 2018) and inflammatory bowel disease (Clerc et al. 2018). Studies have shown patients on antiviral therapy are less likely to receive CV medications (Freiberg et al. 2009), which combined with higher levels systematic of inflammatory biomarkers (GlycA, IgG glycans) could explain the increased risk of CVD in these patients. Research has shown that elevated levels of GlycA are associated with subclinical coronary artery disease in patients with rheumatoid arthritis (Ormseth et al. 2015), psoriasis (Joshi et al. 2016), and lupus (Purmalek et al. 2019). In HIV patients, higher GlycA levels are related to the presence of subclinical coronary artery calcifications, coronary stenosis, and calcified plaque after controlling for levels of other systemic inflammatory markers (Tibuakuu et al. 2019), although this association did not differ by HIV serostatus (Tibuakuu et al. 2019). Alterations in IgG glycans have been monitored to explain CVD, cancer, and other conditions (Lemmers et al. 2017; Vučković et al. 2015; Vučković et al. 2016) in patients with chronic inflammatory diseases (Colomb et al. 2019). Clinical studies have shown that aberrant IgG glycosylation is in direct association with disease onset in these patients (Liu et al. 2018a; Ercan et al. 2010; Rombouts et al. 2015) and that IgG glycans can be used to predict CVD events in patients on antiviral treatments (Liu et al. 2018b; Oswald et al. 2019).

15.3.3 Secondary Prevention of CVD

Secondary prevention aims to reduce the risk of recurrent CVD events and mortality from CVD among patients with established disease. Recent studies suggest that GlycA can also be helpful in identifying individuals with established CVD or with multiple cardiovascular risk factors that are at high risk for complications. In patients with significant CAD at baseline that presented either with acute myocardial infarction or stable angina and underwent cardiac catheterization, elevated GlycA levels are associated with the presence and extent of CAD (McGarrah et al. 2017), future hospitalization related to heart failure (Muhlestein et al. 2018), and increased mortality (Kettunen et al. 2018; McGarrah et al. 2017; Muhlestein et al. 2018). This association is also independent from hsCRP (Muhlestein et al. 2018). Notably, in one of these studies, nondiabetic individuals with very elevated levels of GlycA had a higher risk of all-cause mortality compared to diabetic patients with low GlycA levels (McGarrah et al. 2017), emphasizing the strong association of glycosylation markers with CVD.

15.3.4 Interventions

It is unclear if glycans or glycosylation patterns are directly modifiable by targeted interventions. Despite the positive association between GlycA and modifiable CV risk factors (Akinkuolie et al. 2014), GlycA levels had minimal or clinically not significant decrease in patients receiving statins (Akinkuolie et al. 2016; Sliz et al. 2018) or extended-release niacin therapy added to simvastatin (Otvos et al. 2018). These reports differ from the substantial lowering effects of statins on hsCRP levels, suggesting that there may be new pathways to target low-grade inflammation that could complement statins for the prevention of CVD (Akinkuolie et al. 2016). Similarly, a small association was found between statin use and IgG *N*-glycome (Keser et al. 2017). Promising results have been reported on the effects of diet and lifestyle on glycans. A small intervention study on the effect of repeated sprint training showed an improvement in systemic basal inflammatory IgG *N*-glycosylation profiles compared with controls (Tijardović et al. 2019). Similarly, positive impacts of exercise on reducing GlycA concentration have been reported in another small interventional study (Bartlett et al. 2017). In addition,

modulating IgG glycans through dietary awareness has been observed in patients with cognitive disorders (Witters et al. 2017). Small studies have suggested a possible modulation of glycome after bariatric surgery (Manmadhan et al. 2019) and lifestyle modifications (Olson et al. 2019; Bartlett et al. 2017; Davis et al. 2019), but larger studies need to be performed in order to corroborate this finding. A recent study on a longitudinal TwinsUK cohort showed positive associations between weight loss and IgG glycosylation that indicated supportive effects of changes in lifestyle on reducing immune age (Greto et al. 2020).

One of the emerging approaches for modifying glycans could be anti-inflammatory treatments. In a cohort of patients with psoriasis, GlycA levels improved after antitumor necrosis factor (anti-TNF) treatment, and it was a marker for disease activity (Joshi et al. 2016). A similar positive effect of anti-TNF treatment was observed on IgG *N*-glycosylation markers of inflammation and immune system (Collins et al. 2013; Clerc et al. 2018). Similar trends were seen in a cohort of patients with moderate to severe rheumatoid arthritis that received treatment with Baricitinib (a Janus kinase inhibitor) (Taylor et al. 2018) suggesting that anti-inflammatory treatments could decrease GlycA levels. The use of TNF inhibitors has been shown to improve IgG glycans signatures (Clerc et al. 2018; Ercan et al. 2012; Liu 2015; Atiqi et al. 2020; Bos et al. 2009) in patients with inflammatory diseases. Future studies are needed to see if this decrease in glycan levels would translate into a cardiovascular benefit.

15.4 Summary

In conclusion, glycans capture summative risk related to several inflammatory and immune system pathways, and as such, glycans are promising biomarkers for risk stratification in primary and secondary prediction and prevention of CVD, including in patients with chronic inflammatory conditions. This also supports the potential value of glycan biomarkers and glycomics to identify disease risk pathways and biomarkers especially given the long asymptomatic period of CVD development, where earlier diagnosis could lead to more effective interventions.

Compliance with Ethical Standards

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16

Glycosylation Profiling as a Biomarker of Suboptimal Health Status for Chronic Disease Stratification

Xueqing Wang, Zhaohua Zhong, Lois Balmer, and Wei Wang

Abstract

WHO defines health as "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity." We coined and defined suboptimal health status (SHS) as a subclinical, reversible stage of the pre-chronic disease. SHS is a physical state between health and disease, characterized by health complaints, general weakness, chronic fatigue, and low energy levels. We have developed an instrument to measure SHS, Suboptimal Health Status Questionnaire-25 (SHSQ-25), a self-reported survey assessing five health components that has been validated in various ethnical populations. Our studies suggest that SHS is associated with the major components of cardiovascular health and the early onset of metabolic diseases. Besides subjective measure of health (SHS), glycans are conceived as objective biomarkers of SHS. Glycans are complex and branching carbohydrate moieties attached to proteins, participating in inflammatory regulation and chronic disease pathogenesis. We have been investigating the role of glycans and SHS in multiple cardiometabolic diseases in different ethnical populations (African, Chinese, and Caucasian). Here we present case studies to prove that a combination of subjective health measure (SHS) with objective health measure (glycans) represents a window of opportunity to halt or reverse the progression of chronic diseases.

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Keywords

Glycosylation · *N*-glycan · Suboptimal health status · Biomarkers · Chronic diseases

16.1 Introduction of Suboptimal Health Status

We coined and defined the term "suboptimal health status" (SHS) as an intermediate physical state between health and disease characterized by the perception of health complaints, chronic fatigue, and a constellation of physical symptoms lasting for at least 3 months (Wang and Yan 2012; Wang et al. 2014). The concept of SHS reflects the viewpoint that chronic diseases can be effectively predicted and prevented before the occurrence of a clinical manifestation of severe pathologies (Wang et al. 2016b; Wang et al. 2020; Wang 2020). Recently, SHS has become a global public health challenge (Kupaev et al. 2016; Wang et al. 2016b; Anto et al. 2019; Sun et al. 2020). An increase in awareness of SHS among researchers, patients, and health practitioners is expected to result in an increase in the prevalence of SHS worldwide (Lim et al. 2020). Studies have demonstrated that SHS is associated with an alteration of intestinal microbiota, shortening of telomere length, and reduced mRNA expression level of glucocorticoid receptor α (GRα), plasma cortisol, plasma catecholamines, with an increased incidence of cardiovascular diseases, type 2 diabetes mellitus (T2DM), and preeclampsia (Yan et al. 2015; Alzain et al. 2017; Adua et al. 2017b; Yan et al. 2018; Ge et al. 2019; Anto et al. 2019; Wang et al. 2019; Sun et al. 2020). Therefore SHS, the subclinical, reversible stage of the pre-chronic disease, is the key issue in chronic disease control.

SHS shares similar risk factors with unexplained medical syndrome (UMS), chronic fatigue syndrome (CFS), and myalgic encephalomyelitis (ME) (Münch et al. 1994; Lim et al. 2020). UMS is reported to account for a 20–50% increase in outpatient costs and 30% increase in admission rates in the United Kingdom (Nimmo 2015). In the Australian Federal Pre-Budget 2019–2020, CFS/ME was found to affect more than 240,000 Australians, with a prevalence of 1% (Pre-Budget 2019). Internationally the average prevalence of CFS/ME is 1.46% (Lim et al. 2020). The prevalence of CFS in specific populations, such as nurses, is higher (1.62%) than that in the general population (Lim et al. 2020).

We developed and validated a comprehensive questionnaire, Suboptimal Health Status Questionnaire-25 (SHSQ-25), to assess SHS in different ethnical groups: African, Asian, and Caucasian (Table 16.1) (Yan et al. 2009; Yan et al. 2012; Wang et al. 2016b; Kupaev et al. 2016; Adua et al. 2017b; Anto et al. 2019; Sun et al. 2020). The SHSQ-25 instrument encompasses five health domains: chronic fatigue, mental status, digestive tract, and cardiovascular and immune systems (Fig. 16.1a) (Yan et al. 2009; Wang and Yan 2012). The SHSQ-25 instrument accounts for the multidimensionality of SHS by encompassing the five domains with 25 elements (Fig. 16.1 and Table 16.1). Each individual is asked to rate a statement on a five-point Likert-type scale, based on how often the individual suffers that complaint in the preceding 3 months: (1) never or almost never, (2) occasionally, (3) often, (4) very often, and (5) always. The raw scores of 1 to 5 on the questionnaire are recoded as 0 to 4. SHS scores are calculated for each individual by summing the ratings for the 25 elements. The multidimensional structure of SHSQ-25 has been further assessed by confirmatory factor analysis (CFA) for validity, liability, and repeatability (Fig. 16.1b) (Wang and Yan 2012).

Compared to many survey instruments and risk prediction models (Kolberg et al. 2009; Kahn et al. 2009), the SHSQ-25 instrument is simple and cost-efficient and can be self-completed prior to, or administrated during, a consultation. The scoring system is easy, and data interpretation/analysis does not require special expertise (Yan et al. 2009; Wang and Yan 2012; Adua et al. 2017b). While recognizing this, SHS is a subjective health measure and should be supported with objective biomarkers. Highly sophisticated and powerful analytical tools are available for mea-

Table 16.1 Suboptimal health status questionnaire-25 (SHSQ-25)

In the preceding 3 months, how often was it that you (your)…	1 Never or almost never	2 Occasionally	3 Often	4 Very often	5 Always	Score
1. Were exhausted without greatly increasing your physical activity?						
2. Experienced fatigue that could not be substantially alleviated by rest?						
3. Were lethargic when working?						
4. Suffered from headaches?						
5. Suffered from dizziness?						
6. Eyes ached or were tired?						
7. Suffered from a sore throat?						
8. Muscles or joints felt stiff?						
9. Have pain in your shoulder/neck/waist?						
10. Have a heavy feeling in your legs when walking?						
11. Felt out of breath while sitting still?						
12. Suffered from chest congestion?						
13. Were bothered by heart palpitations?						
14. Appetite was poor?						
15. Suffered from heartburn?						
16. Suffered from nausea?						
17. Could not tolerate cold environments?						
18. Had difficulty falling asleep?						
19. Had trouble with waking up during the night, i.e., kept waking up at night?						
20. Had trouble with your short-term memory?						
21. Could not respond quickly?						
22. Had difficulty concentrating?						
23. Were distracted for no reason?						
24. Felt nervous or jittery?						
25. Caught a cold in the past 3 months?						
Total						

These questions inquire about health events occurring during the past 3 months. Every question is required to be marked with an "x" in the appropriate box, and then the scores are totaled for a SHS score (Yan et al. 2009; Wang and Yan 2012)

suring, detecting, and characterizing biomarkers (Wang et al. 2016a; Wang et al. 2016c; Adua et al. 2017c) aiding in the understanding of the molecular intricacies that underpin the disease' pathogenesis. For example, it is possible to determine transcriptional regulation, posttranslational modifications, altered enzyme activity, and protein expression and interaction (Wang and Jia 1987; Huang et al. 2003; Wang et al. 2016a; Adua et al. 2017c). Researchers have commenced such research where glycosylation profiles are examined in metabolic syndromes (MetS) and T2DM (Lu et al. 2011; Adua et al. 2019b).

16.2 Profiling Glycosylation as Biomarker for Health Measure

Glycosylation, the covalent attachment of sugar moieties (glycans) to proteins, is an important process that takes place in the endoplasmic retic-

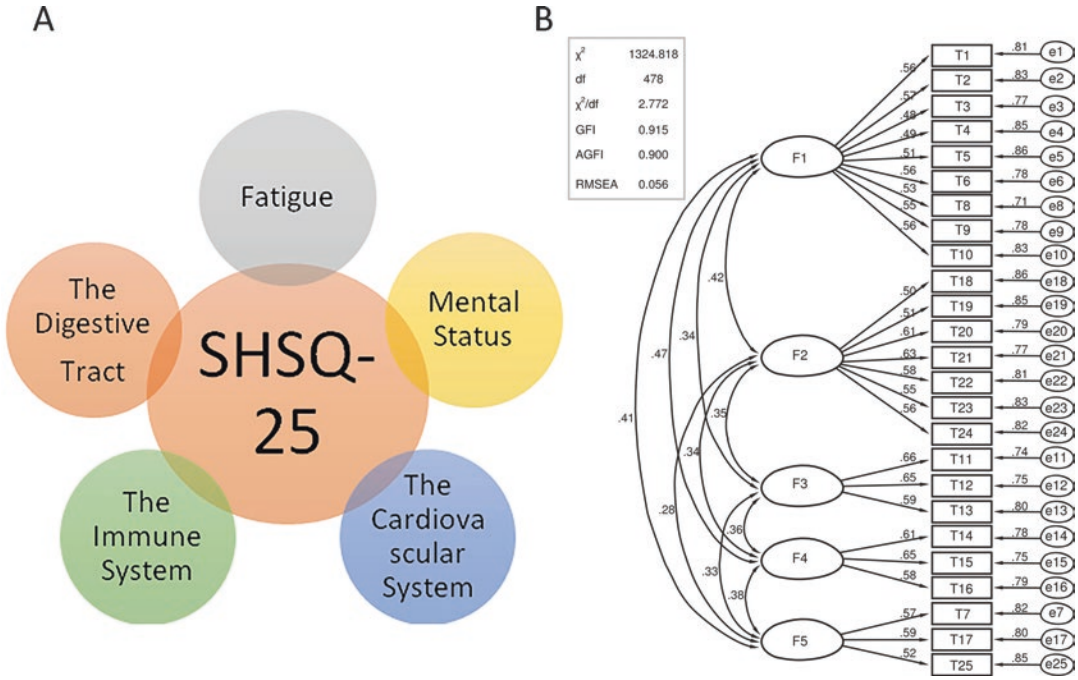
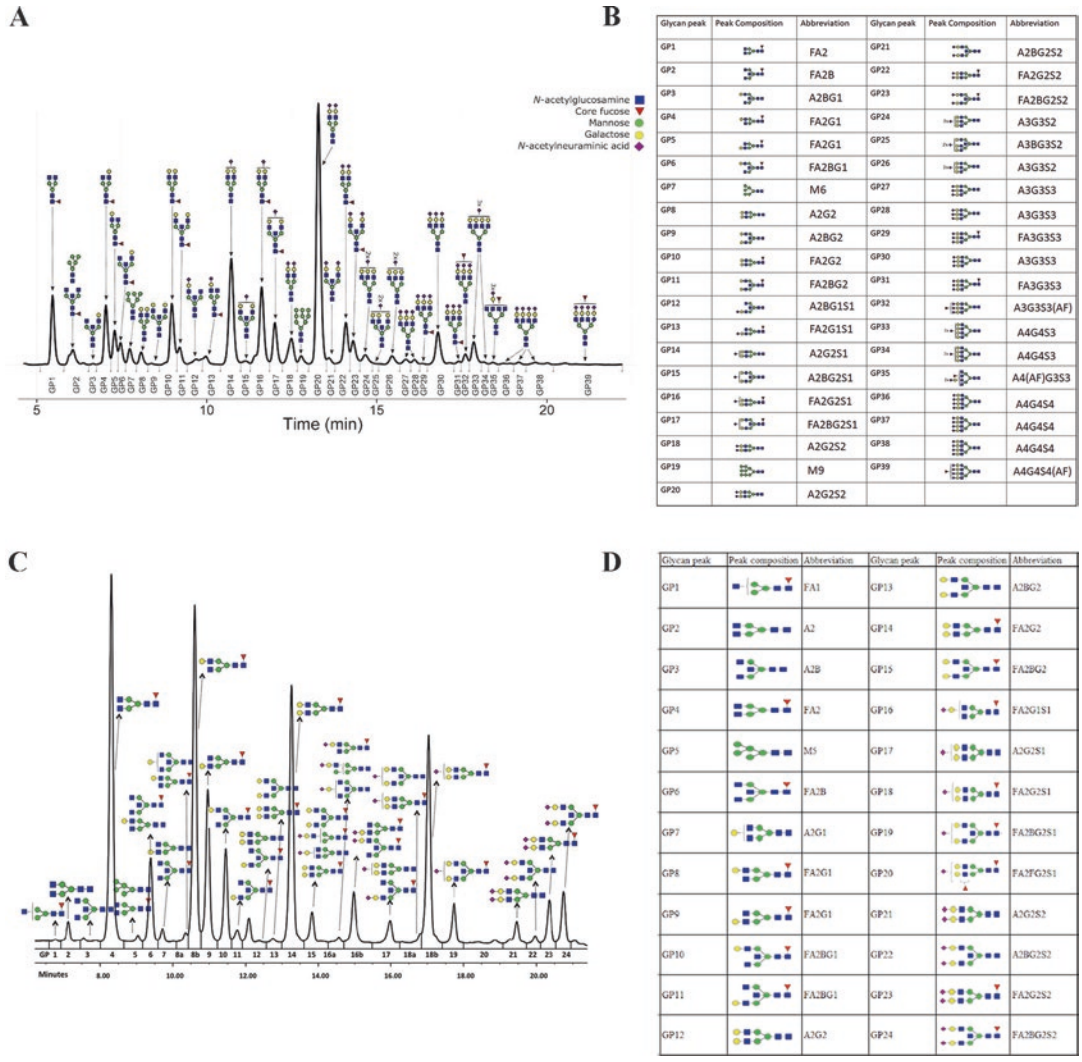


Fig. 16.1 Five domains and 25 elements of the SHSQ-25. (**a**) SHSQ-25 assesses five domains of health: fatigue, cardiovascular function, digestion, immune function, and mental status. (**b**) Confirmatory analysis of the 5 domains and 25 elements of the SHSQ-25. Circles represent the latent variables (5 health domains), and boxes represent each observed variable (25 elements). Values in the middle of two arrowhead lines represent the correlation between domains. Values in the arrows pointing from the domains to the observed values represent loadings of each one of the observed values in the corresponding domain. The values above each observed variable represent the variance explained by the domain. *F1* fatigue, *F2* mental status, *F3* cardiovascular system, *F4* digestive tract, *F5* immune status, *T1–T25* 25 elements in SHSQ-25, *AGFI* adjusted goodness-of-fit index, *df* degree of freedom, *GFI* goodness-of-fit index, *RMSEA* root-mean-square error of approximation

ulum (ER) and the Golgi apparatus (Wang et al. 2016a). The majority of proteins within a cell undergo such a modification. When bound to proteins, glycans affect the protein structure, function, stability, folding, half-life, trafficking, solubility, and their interactions with other cellular molecules (Wang et al. 2016c; Adua et al. 2017c). Based on how glycans are bound to proteins, glycosylation can be classified into (1) *N*-glycosylation, (2) *O*-glycosylation, (3) *C*-glycosylation, (4) glypiation, and (5) phosphoglycosylation (Wang and Özdemir 2019; Wang 2019).

Unlike proteins whose formation follows the transcription and translation of genes (i.e., the central dogma), glycans are formed without requiring a direct template, and their synthesis involves numerous enzymes that add or subtract monosaccharide units to proteins. As a result, the glycosylation process is now associated with the new term "paracentral dogma" (Ma et al. 2018). Although this concept and process may look cumbersome and complex because of the multiple enzymes involved, it is a highly ordered process, and each enzyme is encoded by glycogenes (Wang and Jia 1987; Huang et al. 2003). Hence a disruption of glycogenes or a deficiency of any of the enzymes can lead to a condition commonly termed "congenital disorders of glycosylation" (CDG) (Wang et al. 2016a).

Apart from CDG, evidence from the literature shows that glycans are highly dynamic and their structures change in response to biological and environmental triggers as well as disease presence. For example, changes in glycan structure



Glycan peak	Peak Composition	Abbreviation	Glycan peak	Peak Composition	Abbreviation
GP1		FA2	GP21		A2BG2S2
GP2		FA2B	GP22		FA2G2S2
GP3		A2BG1	GP23		FA2BG2S2
GP4		FA2G1	GP24		A3G3S2
GP5		FA2G1	GP25		A3BG3S2
GP6		FA2BG1	GP26		A3G3S2
GP7		M6	GP27		A3G3S3
GP8		A2G2	GP28		A3G3S3
GP9		A2BG2	GP29		FA3G3S3
GP10		FA2G2	GP30		A3G3S3
GP11		FA2BG2	GP31		FA3G3S3
GP12		A2BG1S1	GP32		A3G3S3(AF)
GP13		FA2G1S1	GP33		A4G4S3
GP14		A2G2S1	GP34		A4G4S3
GP15		A2BG2S1	GP35		A4(AF)G3S3
GP16		FA2G2S1	GP36		A4G4S4
GP17		FA2BG2S1	GP37		A4G4S4
GP18		A2G2S2	GP38		A4G4S4
GP19		M9	GP39		A4G4S4(AF)
GP20		A2G2S2			

Glycan peak	Peak composition	Abbreviation	Glycan peak	Peak composition	Abbreviation
GP1		FA1	GP13		A2BG2
GP2		A2	GP14		FA2G2
GP3		A2B	GP15		FA2BG2
GP4		FA2	GP16		FA2G1S1
GP5		M5	GP17		A2G2S1
GP6		FA2B	GP18		FA2G2S1
GP7		A2G1	GP19		FA2BG2S1
GP8		FA2G1	GP20		FA2FG2S1
GP9		FA2G1	GP21		A2G2S2
GP10		FA2BG1	GP22		A2BG2S2
GP11		FA2BG1	GP23		FA2G2S2
GP12		A2G2	GP24		FA2BG2S2

Fig. 16.2 Chromatograms and structures of glycans separated by HILIC-UPLC. (**a**) All 39 whole plasma protein *N*-glycan peaks (plasma GPs) were generated by HILIC-UPLC, and the sugar moiety is shown above the peak. (**b**) Structures of the 39 whole plasma protein glycans. (**c**) All 24 immunoglobulin G (IgG) *N*-glycan peaks (IgG GPs) were generated on the HILIC-UPLC (**d**) Structures of the 24 IgG glycans. Peak composition is defined as follows: blue square, *N*-acetylglucosamine (GlcNAc); green circle, mannose; red triangle, core fucose/antennary fucose; yellow circle, galactose; purple rhomb, sialic acid. *AF* antennary fucose, *GP* glycan peak, *HILIC-UPLC* hydrophilic interaction ultra-high-performance liquid chromatography, *F* fucose, *A* number of antennas attached to the core sequence (existing of two GlcNAc and three mannose residues), *B* bisecting GlcNAc, *G* galactose, *S* sialic acid

have been linked with the pathophysiology of autoimmune, metabolic, and cardiovascular diseases, including MetS (Lu et al. 2011), cancer (Wang 2013), Parkinson's disease (Russell et al. 2014; Russell et al. 2017), systemic lupus erythematosus (Vučković et al. 2015), rheumatoid arthritis (Sebastian et al. 2016), dyslipidemia (Liu et al. 2018a), hypertension (Liu et al. 2018b), T2DM (Ge et al. 2018), stroke (Liu et al. 2018b), and aging (Yu et al. 2016).

We present the studies on whole plasma protein *N*-glycosylation and immunoglobulin G (IgG) *N*-glycosylation (Fig. 16.2), and their involvement in SHS and SHS-related chronic

diseases. The link between SHS and *N*-linked glycans is mainly examined using hydrophilic interaction ultra-high-performance liquid chromatography (HILIC-UPLC) (Adua et al. 2017c; Liu et al. 2020). IgG is an important effector glycoprotein linking the innate and adaptive branches of the immune system (Liu et al. 2019b), with the ability to exert both pro- and anti-inflammatory responses throughout the body. These immune responses are largely modulated by the fragment crystallizable (Fc) domain of the IgG glycoprotein (Russell et al. 2018). It has been established that the IgG Fc sugar moieties, hereon known as *N*-glycans, affect the affinity of the Fc domain with different Fc receptors, ultimately initiating different cellular events that lead to an array of inflammatory responses, further resulting in disease status (Russell et al. 2018; Liu et al. 2019b).

The whole plasma protein *N*-glycome can be separated into 39 plasma glycan peaks (GPs) (plasma GP1–GP39) (Fig. 16.2a). From these 39 directly measured plasma GPs, an additional 22 plasma glycan traits can be derived (Dagostino et al. 2017; Adua et al. 2019a).

The IgG *N*-glycome can be separated into 24 IgG GPs (IgG GP1–GP24) (Fig. 16.2c). Based on these 24 directly measured GPs, an additional 54 IgG glycan traits can be derived. These GPs describe the level of galactosylation, sialyation, fucosylation, and the degree of branching (Fig. 16.2b and Fig. 16.2d) (Liu et al. 2020).

SHS has become a new public health challenge for psychosocial stress and chronic disease worldwide (Barber et al. 2009; Wang and Yan 2012; Adua et al. 2017b). Identifying the association between SHS and chronic disease risk factors to biological mediators will not only help with effective interventions to reverse SHS but to clarify the mechanisms that mediate the etiological link between SHS and chronic disease. As one of the most extensive, complex, and important posttranslational modifications, glycosylation may provide more information about the mechanisms of SHS and SHS-related chronic disease that can then lead to a reversal in SHS (Adua et al. 2017c; Russell et al. 2018).

16.3 Subjective Measures (SHS) Versus Objective Measures (*N*-Glycans) in Preventive, Predictive, and Personalized Medicine for Chronic Diseases

Researchers are constantly searching for disease-specific biomarkers or predictors for common chronic diseases. Combining subjective and objective measures provides a novel direction for the promotion of diagnostic and candidate biomarkers, without a significant amount of time and resources invested in large-scale population-based investigations.

Our team has been investigating subjective measure (SHS) and objective measure (*N*-glycans) in chronic diseases, including MetS, cardiovascular diseases (CVDs), T2DM, CVD/T2DM comorbidities, autoimmune diseases, neurological disorders, and abnormal senescence (Table 16.2). For these chronic diseases, both SHS and *N*-glycan measures play important roles in the prediction of disease pathogenesis and progression. The integration of subjective measures (SHSQ-25) and objective measures (*N*-glycans) could contribute to progressing towards predictive and personalized/precision medicine for these chronic diseases.

16.3.1 Case Studies for Metabolic Syndrome

16.3.1.1 Case One: Whole Plasma Protein *N*-Glycans as Biomarkers for Metabolic Syndrome in Three Ethnical Populations – Chinese Han, Croatian, and Orcadian

To investigate the correlation between plasma *N*-glycan structures and metabolic syndrome (MetS), we conducted a population-based study in 212 Chinese Han individuals (Lu et al. 2011). Furthermore, a replication study was performed on 520 unrelated individuals from Croatia (Lu et al. 2011).

Table 16.2 Characteristics of included studies

Methodology	Author	Year	Population	Phenotype	Number of all case/control	Glycan peaks (GPs)
IgG *N*-glycosylation profiling using HILIC-UPLC	Vučković et al.	2015	African Caribbean, Chinese Han and Latin American Mestizo	SLE	261/247	IgG GPs 1, 2, 4–12, 14–19, 21–24
	Yu et al.	2016	Chinese Han	Aging	701	IgG GPs 2, 4, 6, 8, 10, 11, 13, 14, 18, 23
	Wang et al.	2016c	Chinese, Croatian and Scottish	Hypertension	4757	IgG GPs 12, 14
	Sebastian et al.	2016	Chinese Han	RA	128/195	IgG GP1
	Russell et al.	2017	Australian and Croatian	Parkinson's Disease	94/102	IgG GPs 5, 8, 14, 17, 20–22
	Liu et al.	2018a	Chinese Han	Dyslipidaemia	598	IgG GPs 4, 6, 9–12, 14, 17, 18, 23
	Liu et al.	2018b	Chinese Han	Ischemic Stroke	78 (IS)/75 (CAS)/77 (Control)	IgG GPs 1, 5, 8, 13–15, 17, 18, 20, 21, 23
	Zhao et al.	2018	Chinese Han	Non-alcoholic Fatty Liver Disease	500	IgG GPs 14
	Russell et al.	2018	Australian	Central Adiposity	637	IgG GPs 1, 3, 4, 7–9, 12–15, 17–22, 24
	Ge et al.	2018	Chinese Han	T2DM	76/435	IgG GPs 3, 4, 8, 11, 14
	Liu et al.	2019a	Chinese Han	Central Adiposity	1196	IgG GPs 4, 5, 7, 10, 11, 13, 15, 17–20, 23, 24
	Liu et al.	2019a, b, c	Australian	T2DM	217/632	IgG GPs 6 and 8
	Wang et al.	2019	Chinese Han	Cardiometabolic risk factors	701	IgG GPs 16,21,18
	Hou et al.	2019	Chinese Han	Hyperuricemia	635	IgG GPs 1, 2, 4, 6, 10–15, 18, 20
	Wang et al.	2020	Chinese Han	MetS	701	IgG GPs 16, 18, 21

(continued)

Table 16.2 (continued)

Methodology	Author	Year	Population	Phenotype	Number of all case/control	Glycan peaks (GPs)
IgG subclass-specific *N*-glycosylation profiling using robust nano-reverse phase LC – MS	Gao et al.	2017	Kazakh	Hypertension	150	IgG1-G1FNS, IgG1-G2FNS, IgG2/3-G0F, IgG2/3-G1FN, IgG2/3-G1FNS, IgG2/3-G2F, IgG2/3-G2N, IgG4-G0F, IgG4-G0FN, IgG4-G1, IgG4-G1N, IgG4-G2, IgG4-G2FS, and IgG4-G2N
	Liu et al.	2018c	Uyghur, Kazakh, Kirgiz and Tajik	Hypertension	274/356	IgG1-G0F, IgG2-G0F, IgG2-G1FN, IgG2-G1FS, IgG2-G2S, IgG4-G0F, IgG4-G1FS, IgG4-G1S, IgG4-G2FS and IgG4-G2N
	Liu et al.	2019c	Uyghur	T2DM	115/122	IgG1-G1, IgG1-G1FS, IgG1-G2FNS, IgG1-G2FS, IgG2-G0, IgG2-G0FN, IgG2-G1, IgG2-G1F, IgG2-G1FS, IgG2-G1N, IgG2-G1S, IgG2-G2, IgG2-G2F, IgG2-G2FN, IgG2-G2FNS, IgG-G2N, IgG2-G2S, IgG4-G0F, IgG4-G0FN, IgG4-G1FN, IgG4-G1N, IgG4-G1NS, IgG4-G1S, IgG4-G2FNS, IgG4-G2N, IgG4-G2NS and IgG4-G2S
Plasma protein *N*-glycosylation profiling using HILIC-UPLC	Adua et al.	2018	African	T2DM progressing to Kidney Disease	241	Plasma GPs 10, 16, 22
	Adua et al.	2019b	African	SHS and MetS	262	Plasma GPs 8, 18, 21, 34
	Adua et al.	2019a	African	T2DM	232/219	Plasma GPs 1, 4, 7, 11, 17, 19, 22, 26, 29

Disease-associated glycan peaks are summarized. *CAS* cerebral arterial stenosis, *GPs* glycan peaks, *HILIC-UPLC* hydrophilic interaction ultra-high-performance liquid chromatography, *LC-MS* liquid chromatography-mass spectrometry, *IS* ischemic stroke, *MetS* metabolic syndrome, *RA* rheumatoid arthritis, *SHS* suboptimal health status, *SLE* systemic lupus erythematosus, *T2DM* type 2 diabetes mellitus, *F* fucose, *N* *N*-acetylglucosamine (GlcNAc), *G* galactose, *S* sialic acid

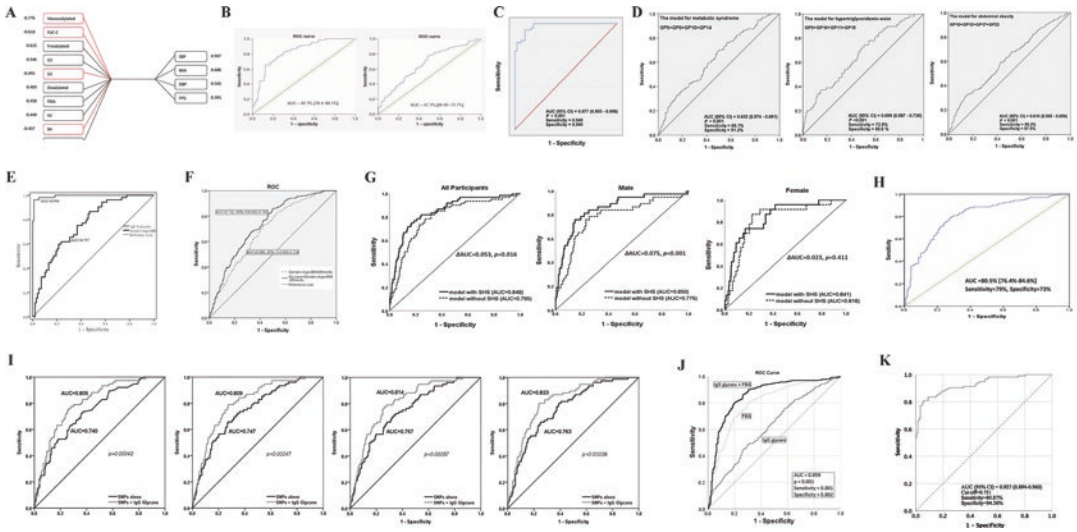


Fig. 16.3 Receiver operating characteristics curves and canonical structures for the case studies. (**a**) Canonical structures of human plasma *N*-glycan- and MetS-related risk factors in a Chinese population. The absolute value of canonical loadings greater than 0.3 with significant loadings. All variables are sorted by absolute value of their canonical loadings. The positive relationship is expressed in black boxes, while a negative relationship is shown in red boxes. (**b**) Performance of the age- and sex-adjusted logistic regression model in predicting the status of patients with (left) metabolic syndrome as a binary outcome and (right) suboptimal health status as a binary outcome in a Ghanaian population. (**c**) The diagnostic performance of potential metabolic biomarkers for SHS in a Chinese population. Four metabolic biomarkers (sphingosine, pregnanolone, taurolithocholate sulfate, and cervonyl carnitine) were selected in the classification model. (**d**) Performance of classification models of (left) metabolic syndrome, (middle) hypertriglyceridemic waist phenotype, and (right) abdominal obesity in a Chinese population. (**e**) Performance of models in predicting hypertension in a Kazakh population. (**f**) Performance of the models in diagnosing hypertension in four ethnic groups. (**g**) Performance of different T2DM predictive models in a Chinese population. Model constructed with/without SHS among (left) all participants, (middle) male participants, and (right) female participants. (**h**) Performance of the age- and sex-adjusted logistic regression model in predicting the status of patients with T2DM and healthy controls in a Ghanaian population. (**i**) Performance of (from left to right) crude model (no adjustment for covariates); adjusted for age and gender; adjusted for age, gender, and BMI; and adjusted for age, gender, BMI, SBP, DBP, HDL-C, LDL-C, and WHR in a Chinese population. (**j**) Performance of the prediction model of T2DM in an Australian population. (**k**) Performance of the classification model in delineating T2DM in an Uyghur population. (**k**) *AUC* area under the curve, *BMI* body mass index, *DBP* diastolic blood pressure, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *ROC* receiver operator characteristic, *SBP* systolic blood pressure, *WHR* waist-to-hip ratio

The most prominent observation was the simultaneously consistent positive correlation between different forms of triantennary plasma *N*-glycans and negative correlation between plasma *N*-glycans containing core fucose with MetS components: body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), and fasting blood glucose (FBG). Significant differences in plasma *N*-glycan traits were also detected between normal and abnormal groups of BMI, blood pressure, and FBG, respectively. Among the 10 *N*-glycan structures (monosialylated, core-fucosylated, trisialylated, trigalactosylated, digalactosylated, disialylated, triantennary, biantennary agalactosylated, biantennary, and agalactosylated glycans), multivariate analysis revealed that the level of monosialylated glycans (structure loadings = −0.776) was the most correlated with MetS-related risk factors, especially with SBP (structure loadings = 0.907) (Fig. 16.3a).

Since these results were obtained from a cross-sectional study, further case-control or cohort studies with large samples are warranted to provide a more definite explanation about the relationship between plasma protein *N*-glycan

structures and MetS. A case-control study recruiting 2155 individuals (564 MetS cases and 1475 healthy controls) from the Orkney Islands (United Kingdom) was conducted to verify the role of plasma *N*-glycans in MetS (McLachlan et al. 2016).

A total of 12 plasma *N*-glycan traits (especially monosialylation) were found to be associated with an increased likelihood of having MetS, while 9 plasma *N*-glycan traits (especially core fucosylation) were negatively associated with MetS. Notably, several statistically significant associations between plasma *N*-glycans and the components of BMI, blood pressure (BP), and FBG which had been identified in Chinese and Croatian populations were found to be replicated in this Orcadian study. The consistency demonstrated a protective association of higher levels of core-fucosylated plasma *N*-glycans. These results showed that variations in the composition of the plasma *N*-glycome could represent the alternations of human metabolism and could be potential biomarkers of MetS.

16.3.1.2 Case Two: Whole Plasma Protein *N*-Glycans as Risk Stratification Biomarkers for SHS and MetS in a Western African Ghanaian Population

To further validate the role of plasma protein *N*-glycans in MetS and to investigate the interplay between MetS and SHS, 262 participants were recruited in Ghana, Western Africa (Adua et al. 2019b). In order to screen for individuals with undiagnosed risk factors, we excluded participants who had been previously diagnosed with diabetes and/or hypertension and who were suffering from other chronic diseases (Adua et al. 2019b).

Four plasma glycan peaks representing galactosylation and sialylation were found to predict MetS and SHS using a logistic regression model (Table 16.2). This model yielded an AUC of MetS 83.1% (95% CI: 78.0–88.1%) and SHS 67.1% (60.6–73.7%) (Fig 16.3b). These results indicated that plasma *N*-glycome could be a significant, albeit modest, risk factor for MetS and SHS.

16.3.1.3 Conclusions for Case Studies of Metabolic Syndrome

Plasma protein *N*-glycan structural features have been found to be significantly correlated with MetS risk factors, including age, BMI, blood pressure, and FBG. Furthermore, plasma *N*-glycan profiles are promising biomarkers for SHS and MetS prediction. Plasma protein *N*-glycans and plasma metabolites, such as sphingolipids, have also been used as valuable biomarkers for SHS identification (Fig 16.3c) (Wang et al. 2020). SHS-related metabolic disturbances could be detected at the early onset of SHS and the progression towards chronic cardiometabolic diseases.

Therefore, MetS and SHS with *N*-glycans could create a window opportunity for a personalized precision medication of metabolic diseases. The integration of subjective health measure (SHS) and objective health measure (*N*-glycome and metabolites) would enable for targeted prevention before the onset of chronic diseases.

16.3.2 Case Studies for Cardiovascular Diseases

16.3.2.1 Case One: IgG *N*-Glycans as Biomarkers for Cardiometabolic Health in a Chinese Han Population

Cardiovascular disease is a common complex trait that calls for next-generation objective biomarkers for precision diagnostics and therapeutics. Our previous studies demonstrated that IgG *N*-glycans affect the anti- and pro-inflammatory responses of IgG and are associated with cardiometabolic risk factors such as aging, central obesity, dyslipidemia, and hyperglycemia (Yu et al. 2016; Alzain et al. 2017; Gudelj et al. 2018; Liu et al. 2018a; Ge et al. 2018; Russell et al. 2019; Liu et al. 2019a).

Investigation of the role of IgG *N*-glycans in cardiovascular disease identified an association

between IgG *N*-glycans with 15 cardiometabolic risk factors in a community-based cross-sectional study with 701 Chinese Han participants (Wang et al. 2019).

After controlling for age and sex, IgG *N*-glycans FA2G1S1, A2G2S2, and FA2G2S1 were significantly different in participants with and without MetS, hypertriglyceridemic waist phenotype, or abdominal obesity, respectively. These three glycans presented sialylation with monogalactosylation or digalactosylation. A canonical correlation analysis showed that IgG *N*-glycan profiles were significantly associated with cardiometabolic risk factors ($r = 0.469$, $p < 0.001$). Classification models based on IgG *N*-glycan traits were able to differentiate participants with (1) MetS, (2) hypertriglyceridemic/waist phenotype, or (3) abdominal obesity from controls, with an AUC of 0.632 (95% CI: 0.574–0.691), 0.659 (0.587–0.730), and 0.610 (0.565–0.656), respectively (Fig 16.3d).

These results suggested that IgG *N*-glycans might play an important role in cardiometabolic disease pathogenesis by regulating the pro- and anti-inflammatory responses of IgG.

16.3.2.2 Case Two: Association Between IgG Glycosylation and Hypertension in Three Ethnical Populations – Chinese Han, Croatian, and Scottish

A multicenter cross-sectional study was carried out to investigate the association between hypertension and IgG *N*-glycosylation (Wang et al. 2016c). A total of 4757 individuals of Chinese Han, Croatian, and Scottish ethnicity were recruited for this study.

Five IgG *N*-glycans (IgG with agalactosylated glycans) were significantly associated with pre-hypertension or hypertension compared to those with normal blood pressure, while an additional 17 IgG *N*-glycan traits (majority with fucosylation) were associated in participants with hypertension compared to those of normal blood pressure. These IgG *N*-glycans were also significantly correlated with SBP or DBP (Wang et al. 2016c).

These results demonstrate an association between hypertension and IgG *N*-glycome composition. These findings suggest that the individual variation in *N*-glycosylation of IgG contributes to the pathogenesis of hypertension, presumably via its effect on inflammatory regulation.

16.3.2.3 Case Three: IgG Subclass *N*-glycans as Biomarkers for Hypertension in Multiple Chinese Minority Ethnical Populations – Kazakh, Uygur, Kirgiz, and Tajik

We have further demonstrated that IgG subclass-specific *N*-glycome composition is associated with hypertension. The subclass-specific IgG *N*-glycans were further verified as potential biomarkers for hypertension when investigated with a cohort of 150 Kazakh participants, a minority ethnic group, resident in the western frontier area of China (Gao et al. 2017). The IgG glycome profile on the level of 60 subclass-specific *N*-glycopeptides was analyzed by nanoLC coupled to mass spectrometry (MS). Fourteen IgG subclass-specific Fc *N*-glycopeptide structures, along with one derived IgG *N*-glycan trait, were found to correlate with SBP and/or DBP (Table 16.2). These glycan traits primarily presented with fucosylation and galactosylation. The receiver operating characteristic (ROC) curve analysis showed that the performance of the model including a selection of 9 IgG *N*-glycans was greater than the traditional model based on gender, age, and BMI for differentiation of hypertension and healthy controls in the Kazakh population sample (AUC = 0.994, 95% CI: 0.985–1.000; AUC = 0.757, 95% CI: 0.679–0.835) (Fig 16.3e).

Furthermore, IgG subclass-specific *N*-glycosylation has been used as a method to profile biomarkers in multiple minority populations in China (Liu et al. 2018c). A total of 274 hypertensive patients and 356 healthy controls from 4 northwestern Chinese minority populations, including Uygur (UIG), Kazakh (KZK), Kirgiz (KGZ), and Tajik (TJK), were recruited. The results showed that 10 directly measured IgG

N-glycan traits, which included galactosylation and sialylation, are significantly associated with hypertension. Two different models were able to be constructed from this data using logistic regression (Fig 16.3f). The AUC of 0.688 (95% CI: 0.650–0.724) in the baseline model utilized gender, age, BMI, and ethnicity as the covariates, and 0.732 (95% CI: 0.696–0.766) in the glycan-based model utilized 5 significantly different IgG subclass-specific *N*-glycans together with the aforementioned covariates, respectively. The AUCs showed modest improvement in the glycan-based diagnostic model compared to the baseline model, with an increment of 0.044 (95% CI: 0.016–0.072).

N-glycome of IgG subclasses is associated with blood pressure status in multiple Chinese northwestern minority populations, including UIG, KZK, KGZ, and TJK, and therefore offers the opportunity across different ethnical groups for personalized medical management of hypertension.

16.3.2.4 Conclusions for Case Studies of Cardiovascular Diseases

These studies suggest that SHS is associated with the majority of components of cardiovascular health (CVH) metrics defined by the American Heart Association (AHA) (Lloyd-Jones et al. 2010; Yan et al. 2012; Kupaev et al. 2016). We investigated the association between SHS and CVH metrics in a cross-sectional analysis of China suboptimal health cohort study (COACS) (Wang et al. 2017). A total of 4 CVH metrics (smoking, physical inactivity, poor dietary intake, and ideal control of blood pressure) are significantly correlated with the risk of poor SHS. In addition to the SHS score, specific IgG *N*-glycan traits, including galactosylation and sialylation, are significantly associated with CVH metrics in multiple ethnic populations. IgG *N*-glycans may participate in the pathogenesis of cardiovascular diseases by regulating the pro- or anti-inflammatory response of IgG. Therefore, the evaluation of SHS combined with the analyses of IgG *N*-glycome would allow a more precise risk classification of CVDs, which would contribute to the personalized precision medication of CVD-related chronic diseases.

16.3.3 Case Studies for Type 2 Diabetes Mellitus

16.3.3.1 Case One: SHS as an Independent Risk Factor for Type 2 Diabetes Mellitus in a Chinese Han Population

A prospective longitudinal cohort study was conducted based on the COACS project to understand the impact of SHS on the progressing of T2DM (Ge et al. 2019). An association between SHS and T2DM was examined using multivariable logistic regression models and predictive models for T2DM onset based on SHS.

A total of 61 participants developed T2DM approximately 3 years after follow-up. Participants with higher SHS score had a higher incidence of developing T2DM ($p = 0.036$). Moreover, compared with the lowest SHS score, participants with higher SHS scores were found to be associated with 1.6-fold risk of developing T2DM. The predictive model constructed with SHS had higher discriminatory power (AUC = 0.848) than the model without SHS (AUC = 0.795) (Fig 16.3g).

A higher SHS score is associated with a higher incidence of T2DM. The study shows that SHS is a new independent risk factor for T2DM and has the capability to act as a predictive tool for T2DM onset. The evaluation of SHS combined with the analysis of modifiable risk factors for SHS allows the risk stratification of T2DM, which may consequently contribute to the prevention of T2DM development.

16.3.3.2 Case Two: Glycans and SHS as Potential Risk Assessments for Type 2 Diabetes Mellitus in a Western African Ghanaian Population

A further study examined various factors that characterize SHS and T2DM in Ghana, Western Africa (Adua et al. 2017b). Using a cross-sectional design, we recruited 241 T2DM patients and 264 people as controls. The controls were categorized into high and low SHS based on the SHSQ-25 score.

Sedentariness, SBP and DBP were significantly correlated with SHS. After adjusting for age and gender, central adiposity, underweight, high SBP, high DBP, and high triglyceride (TG) were found to be independent risk factors associated with SHS.

The management of T2DM in Ghana is suboptimal, and undiagnosed risk factors remain prevalent. These results show that the SHSQ-25 instrument could be translated and applied as a practical tool to screen at-risk individuals and hence prove useful for preventing T2DM in the economically underdeveloped areas (Adua et al. 2017b).

To investigate the association between plasma *N*-glycans with T2DM in this underprivileged region, Ghana, a case-control study was conducted (Adua et al. 2019a). Whole plasma protein *N*-glycans of 232 T2DM patients and 219 healthy controls were analyzed. Nine plasma *N*-glycan peaks representing core fucosylation, galactosylation, or high mannosylation were found to determine and possibly even predict T2DM status (Table 16.2). This prediction model yielded an AUC of 80.5% with a sensitivity of 79% and specificity of 73% (Fig 16.3h), indicating the predictive power of plasma *N*-glycans as robust biomarkers of T2DM in this Ghanaian population.

16.3.3.3 Case Three: IgG *N*-glycans as Biomarker for Type 2 Diabetes Mellitus in Multiple Ethnical Populations – Chinese Han, Uyghur, and Australian

We conducted a case-control study to identify IgG *N*-glycan biomarkers of T2DM in 511 Chinese Han participants (Ge et al. 2018). Among them, 76 had increased FBG (aged 48 ± 6.14), and 435 had decreased or fluctuant FBG (aged 48 ± 6.08).

IgG glycan traits presenting with bisecting *N*-acetylglucosamine (GlcNAc) (A2B, FA2G1, and FA2BG1) linked with a positive association to an increased FBG, whereas galactosylation traits (FA2 and FA2G2) linked with a negative association. In addition, nine DNA single nucleotide polymorphisms (SNPs) were identified to be associated with increased FBG. A significant improvement in the predictive power was observed when including 24 IgG GPs to the existing 9 DNA SNPs, the AUC increased from 0.75 to 0.81 (Fig 16.3i).

A further study in Australia verified the association between IgG *N*-glycan traits, T2DM, and clinical risk factors (Li et al. 2019). A total of 849 participants (217 T2DM cases and 632 healthy controls) were recruited from an urban community in Busselton, Western Australia. A decrease of galactosylation and increase of fucosylation with bisecting GlcNAc (6 GPs) were significantly associated with T2DM, after correction for extensive clinical confounders and false discovery rate (Table 16.2). Moreover, adding the IgG glycan profiles to FBG in the logistic regression model increased the AUC from 0.799 to 0.859. The AUC for IgG glycans alone was 0.623 with a 95% CI of 0.580–0.666 (Fig 16.3j).

For Chinese minority groups, a validation study compared IgG subclass-specific *N*-glycan traits in 115 T2DM patients with 122 healthy controls in a UIG population (Liu et al. 2019c). Twenty-seven directly measured and four derived *N*-glycan traits (majority with galactosylation) of the IgG subclass-specific *N*-glycopeptides were significantly associated with T2DM. A classification model based on IgG subclass-specific *N*-glycan traits was able to distinguish patients with T2DM from controls with an AUC of 0.927 (95% CI: 0.894–0.960) (Fig 16.3k).

These studies report associations between IgG *N*-glycome, especially bisecting GlcNAc and galactosylation, with T2DM in multiple ethnical populations. Genomic meeting glycomic approach contributes to the combination of candidate DNA SNPs with IgG *N*-glycans, which offers novel biomarker potential for the progression of T2DM. The substantial predictive power obtained from integrating multi-omics enables more personalized management of T2DM.

16.3.3.4 Conclusions for Case Studies of Type 2 Diabetes Mellitus

The profiles of IgG *N*-glycosylation and whole plasma protein *N*-glycome are associated with T2DM in different ethnical populations, suggesting a potential for the *N*-glycome as a biomarker candidate for T2DM. The integration of glycom-

ics with other subjective measures, such as SHS, might offer further confidence for innovation in diagnosis and personalized precision medication for T2DM.

16.4 Discussion

We coined and defined SHS as an "intermediate phenotype" that is predictive of long-term adverse health and thus an ideal phase for therapeutic and lifestyle interventions (Yan et al. 2009; Wang and Yan 2012; Wang et al. 2016b). Our data suggests that this intermediate stage represents a window of opportunity to halt or reverse the progression of chronic disease. We propose that IgG *N*-glycome and whole plasma protein *N*-glycome could serve as objective quantitative biomarkers for such an intermediate phenotype of advancing ill health over time, including cardiovascular health metrics (Kupaev et al. 2016), hypertension (Wang et al. 2016c; Gao et al. 2017), stroke (Liu et al. 2018b), T2DM and MetS (Wang et al. 2014; Adua et al. 2017a; Adua et al. 2019b; Ge et al. 2019), aging (Alzain et al. 2017), autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Vučković et al. 2015; Sebastian et al. 2016), neurodegenerative diseases such as Parkinson's disease (Russell et al. 2017), and cancer (Wang 2013).

The book chapter indicates that SHS as well as IgG and whole plasma protein *N*-glycome are associated with multiple cardiovascular health metrics and the development of metabolic diseases (MetS and T2DM). These cardiometabolic diseases are multifactorial driven by diverse pathologies, including genetic variants, epigenetic regulation, posttranslational modifications, acquired environmental factors, and their interactions at multi-omic levels. The glycome reflects the real-time status of these intricate interplays and promises potential biomarkers for multifactorial diseases (Wang et al. 2016a).

These complex and highly specific *N*-glycan residues, including fucose, GlcNAc, mannose, galactose, and sialic acid, reportedly modulate the pro- and anti-inflammatory effects of IgG. Core fucose plays a major role in regulating the magnitude of antibody-dependent cellular cytotoxicity (ADCC), and highly fucosylated IgG shows reduced ADCC (Shinkawa et al. 2003; Liu et al. 2019b). A lack of core fucose leads to a 4- to 100-fold increase in ADCC, making IgG more pro-inflammatory. The addition of GlcNAc to the bisecting mannose of IgG *N*-glycans increases ADCC and the binding affinity to Fcγ receptor IIIA (FcγRIIIA) (Shade and Anthony 2013). Mannose-rich *N*-glycans have an increased affinity for mannose-binding lectin (MBL), which initiates the lectin complement cascade (Fujita 2002). However, studies have shown that IgG with high mannose structures have a negative impact on CDC activity because of lower IgG binding affinity for complement component 1q (C1q) (Kanda et al. 2007; Yu et al. 2012). High mannose glycoforms exhibited higher FcγRIIIA binding and ADCC activity (Yu et al. 2012). The addition of terminal galactosylation to the IgG *N*-glycans can exhibit anti-inflammatory activity (Karsten et al. 2012). Terminal galactosylation enhances the efficacy of C1q binding and complement-mediated cytotoxicity (CDC) (Peschke et al. 2017). Researchers have demonstrated enhanced ADCC upon enzymatic hypergalactosylation of four different monoclonal IgG1 antibodies (Thomann et al. 2016). However, while galactose can influence ADCC activity, lacking core fucosylation remains the primary driver of enhanced ADCC activity. Terminal sialic acid residues in the IgG *N*-glycans additionally mediate galactosylation anti-inflammatory response (Kaneko et al. 2006). IgG Fc sialylation of human monoclonal IgG1 molecules impairs IgG1 efficacy to induce CDC (Quast et al. 2015). The addition of sialic acid inhibits ADCC, and the effect of sialylation on ADCC is dependent on the status of core fucosylation (Li et al. 2017). Terminal sialylation is responsible for anti-inflammatory activity, but the final effect of IgG sialylation depends on the monosaccharide composition of IgG Fc *N*-glycans (Cymer et al. 2018). Therefore, different IgG Fc domain sugar moieties have different effects to regulate the inflammatory function of IgG (Table 16.3).

Table 16.3 Summary of altered IgG glycosylation and its downstream effects

IgG glycan traits	CDC	ADCC	Anti-inflammation
High fucose		↓	↑
Bisecting *N*-acetylglucosamine		↑	
High mannose	↓	↑	
High galactose	↑	↑	↑
High sialic acid	↓	↓	↑

Up arrows represent an increase, down arrows represent a decrease, and blank boxes represent to be confirmed. *ADCC* antibody-dependent cell cytotoxicity, *CDC* complement-dependent cytotoxicity, *IgG* immunoglobulin G

The association of IgG *N*-glycosylation with cardiometabolic diseases implies a pro-inflammatory state in the patients. Our studies in different ethnical populations show that the decreasing galactosylation and sialylation, while increasing bisecting GlcNAc, are risk factors of certain inflammatory and chronic diseases, including hypertension (Wang et al. 2016c; Gao et al. 2017), stroke (Liu et al. 2018b), and T2DM (Adua et al. 2017c; Keser et al. 2017; Adua et al. 2018; Ge et al. 2018; Li et al. 2019; Liu et al. 2019b). Furthermore, we have investigated the involvement of *N*-glycome alterations in other chronic or autoimmune diseases, including dyslipidemia (Liu et al. 2018a), nonalcoholic fatty liver diseases (Zhao et al. 2018), hyperuricemia (Hou et al. 2019), diabetic kidney disease (Adua et al. 2018), Parkinson's disease (Russell et al. 2017), systemic lupus erythematosus (Vučković et al. 2015), and rheumatoid arthritis (Sebastian et al. 2016).

Aberrant glycosylation of IgG was also reported in SHS, abdominal obesity, and aging (Yu et al. 2016; Gudelj et al. 2018; Wang et al. 2019; Adua et al. 2019b; Russell et al. 2019). These reports suggest that individual changes of IgG *N*-glycans might not be disease specific, but an abreaction of profiling of IgG *N*-glycome represents a general health phenotype, such as SHS, associated with an altered inflammatory function of circulating IgG. As a posttranslational modification related to immune function, IgG *N*-glycosylation provides more information about the inflammatory status of health.

Therefore, a combination of subjective health measure, SHS, and objective measure, *N*-glycan, is poised to bring about new insights into dynamic host-environment interactions or the combined effects of both nature and nurture that ultimately underlie the basis of most complex phenotypes. Predictive and personalized glycomedicine is a new discipline that employs glycomic biomarkers in the hopes of better targeting disease diagnostics, drug discovery, prescription choice, and medication dosing based on individual glycomic profiles (Wang et al. 2014; Wang et al. 2016b; Kupaev et al. 2016; Adua et al. 2019b; Wang and Özdemir 2019).

Compliance with Ethical Standards

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Ethical Approval The study cases discussed in this book chapter have the ethical approvals obtained from each of the individual institutions, respectively, so that this book chapter review does not contain any studies with human participants performed by any of the authors.

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Glycosylation and Aging

17

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Abstract

Human lifespan has increased significantly in the last 200 years, emphasizing our need to age healthily. Insights into molecular mechanisms of aging might allow us to slow down its rate or even revert it. Similar to aging, glycosylation is regulated by an intricate interplay of genetic and environmental factors. The dynamics of glycopattern variation during aging has been mostly explored for plasma/serum and immunoglobulin G (IgG) *N*-glycome, as we describe thoroughly in this chapter. In addition, we discuss the potential functional role of agalactosylated IgG glycans in aging, through modulation of inflammation level, as proposed by the concept of inflammaging. We also comment on the potential to use the plasma/serum and IgG *N*-glycome as a biomarker of healthy aging and on the interventions that modulate the IgG glycopattern. Finally, we discuss the current knowledge about animal models for human plasma/serum and IgG glycosylation and mention other, less explored, instances of glycopattern changes during organismal aging and cellular senescence.

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Keywords

Aging · Inflammaging · Glycosylation · IgG *N*-glycome · Plasma *N*-glycome · Biological age

17.1 Introduction

Life expectancy has increased globally by approximately 27 years in the last 70 years, showing a very steep upward trend (Our World in Data 2020a). Due to the very short timeframe on an evolutionary scale, such a dramatic increase cannot be attributed to the forces of natural selection. Instead, it is likely human intervention in the form of economic and medicinal development has ultimately led to a much healthier population, with, consequentially, a longer lifespan (Our World in Data 2020a).

In addition to the environmental factors that are largely dependent on lifestyle and economic status, the lifespan is partly associated with genetic makeup. Lifespan heritability, here defined as the proportion of the trait variance that can be attributed to genetic factors, ranges from around 15 to around 30% (Herskind et al. 1996; Graham Ruby et al. 2018).

Healthy Aging The realization that longevity is susceptible to relatively quick changes, fueled by the everlasting human search for immortality,

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rekindled the immense interest in one of biomedicine's grandest goals—prolonging our lifespan. However, there is a catch to this concept. The observed increase in life expectancy means the global population is, on average, getting older (Gliwicz 1983). While western societies are redefining the meaning of certain life stages ("30s are the new 20s," "50s are the new 30s," etc.), increasing age remains one of the biggest risk factors for chronic diseases. This means that simply prolonging the lifespan will not do. We do not just want to live longer, we strive towards a healthy, fulfilled, and active long life. Thus, the quest for immortality has evolved into a quest for life quality.

We appear to have successfully transformed what was once considered "end-of-life" into "the golden years" of adulthood. Will we have to stop here or is there room for further improvement? Is it possible to assess if a person is on the right path towards healthy aging? Can we revert an unhealthy aging process, once underway? What should one do to slow down the rate of aging and thus preserve high quality of life? To answer these questions, a reliable biomarker of healthy aging is necessary.

Glycosylation and Aging The biosynthesis of glycans is not dictated by a direct genetic template but is instead under the control of many genes and various environmental factors (Lauc 2016). The sequence of a glycan, therefore, shows the integrated effect of an individual's genetic makeup and the environment (Lauc 2016), as is the case with aging (Titorenko 2018). Glycosylation changes have been associated with virtually all physiological states, including aging, as reported in numerous studies (Knežević et al. 2009, 2010; Gudelj et al. 2018). Most of them were conducted on plasma/serum and immunoglobulin G (IgG) *N*-glycans, as will be described in detail later.

This chapter will focus on glycosylation changes accompanying aging on an organismal and cellular level, and their potential functional role. We will discuss the performance of plasma and IgG *N*-glycans as biomarkers of healthy aging, and explore the possibility to use them as molecular levers to slow down or revert the rate of aging. We will comment on the aptness of animal models for researching glycosylation in aging, and mention some less explored age-dependent glycopattern changes. Besides that, we will discuss glycosylation changes accompanying cellular senescence and malign transformation.

17.2 What Is Aging?

Human Lifespan We have witnessed a striking prolongation of the human lifespan in the last 200 years—doubling in some countries (UK), tripling, and quadrupling in others (India and South Korea, respectively) (Our World in Data 2020a). Interestingly, in very recent years, there have been instances of an opposite trend in some of the developed countries. For example, the average lifespan of Americans dropped by 0.1 years each year from 2014 to 2017 (Xu et al. 2016; Kochanek et al. 2017; Murphy et al. 2018). This unexpected phenomenon has been attributed to the increased mortality due to age-related pathological conditions such as cerebrovascular and cardiovascular diseases, as well as lung diseases caused by smoking and obesity; but also social factors such as homicides, suicides, drug abuse, road accidents, and lack of universal health care (States 2017; Our World in Data 2020b).

Aging To fathom how to accomplish staying healthy while aging, we first need to understand the fundamentals—the physiological and molecular processes underlying aging. The simplest definition of aging states it is a gradual functional decline of an organism. It is a complex biological phenomenon that entails an alteration of many different processes on the cellular and organismal level. The underlying mechanisms of aging-related processes and conditions are many and not yet fully elucidated. The ones that have been characterized so far include the nine hallmarks of aging: genomic instability, telomere attrition,

epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín et al. 2013; Kennedy et al. 2014). The timing and the extent of the activation of these mechanisms have been correlated with both genes and environment (Titorenko 2018). On the level of a whole organism, these changes impair function and increase susceptibility to death (López-Otín et al. 2013), due to the onset of various age-related physiological states of disturbed homeostasis (Franceschi et al. 2018).

Geriatric Syndromes and Age-Related Diseases The global lifespan increase has given rise to a much higher prevalence of geriatric syndromes (GSs) and age-related diseases (ARDs) (Hajat and Stein 2018). GSs are defined as unique clinical features of common conditions in the elderly that do not fit into discrete disease categories, such as frailty, delirium, falls, dizziness, urinary incontinence, and sarcopenia (Inouye et al. 2007). ARDs are diseases the prevalence of which increases with age, such as cardiovascular diseases, osteoporosis, type II diabetes, and some types of cancer (Franceschi et al. 2018).

The need to understand the exact relationship between aging and the onset of ARDs and GSs has given rise to an interdisciplinary branch of science, called geroscience. Although age research is complex and often results in opposing theories (Hayflick 2007), based on most of the existing epidemiological and experimental data, Franceschi's team proposes that there is little, if any, difference in causative cellular and molecular mechanisms between ARDs or GSs and aging (Franceschi et al. 2018). This implies that people who reach an extremely old age largely avoid or postpone the onset of ARDs and GSs (Franceschi et al. 2018). An interesting shared trait of ARDs and GSs is their gradual and often long-lasting development, so their beginnings are asymptomatic and frequently undiagnosed until the disease has reached an irreversible stage (Franceschi et al. 2018).

Biological Age In contrast to chronological age, which considers solely our date of birth, biological age (also called functional or physiological age) refers to the functional fitness of our organism (Hamczyk et al. 2020). Influenced by genetic and environmental factors (e.g., lifestyle), biological age does not necessarily match our chronological age, with the difference between the two corresponding to the degree of healthy aging (Vilaj et al. 2019; Hamczyk et al. 2020).

The presence of ARDs and GSs increases biological age, lowers life quality, and shortens the healthspan. Healthy aging can thus be defined as reaching old age without developing ARDs or GSs. Currently, due to the complexity of the interplay between genes and the environment, the degree of healthy aging is nearly impossible to predict on an individual level. It is equally difficult to recognize the initiation of the detrimental processes at the root of aging while they remain asymptomatic. Most importantly, by the time symptoms show up, it might be too late for effective preventive measures.

For these reasons, there is a need for a biomarker that determines biological age. The ideal biomarker of biological age, as defined by the American Federation for Aging Research, should predict the rate of aging while monitoring a basic process that underlies aging (not the effect of a disease), should be suited for repeated testing without harming the person, and should work in laboratory animals (Butler et al. 2004; Johnson 2006). Additionally, it should be easily analyzed in a robust, high-throughput manner, have day-to-day stability while remaining sensitive enough to detect changes that occur over a relatively short period of time (e.g., after intervention), and not be extremely sensitive to environmental factors (like seasonality).

Equally important, the pursuit of a predictor of biological age is expected to help elucidate the elementary mechanisms behind the aging process, thus possibly offering a way to intervene and slow down aging on the molecular level.

17.3 Glycosylation and Aging

Glycans cover all eukaryotic cells and were originally thought to adorn more than half of all human proteins, including almost all membrane and secreted proteins (Apweiler et al. 1999; Moremen et al. 2012). A newer study estimates, however, that at best, less than one-fifth of all proteins are glycosylated (Khoury et al. 2011). Either way, glycans participate in a multitude of biological processes that are based on molecular and cellular interactions (Varki 2017), some of which become increasingly deregulated as we age, for example, cell-to-cell communication.

Glycosylation and aging are intricately intertwined, their relation not at all easy to disentangle. As a process that depends on the activity of a multitude of enzymes, accessibility of their substrates, cell metabolism, and homeostasis, glycosylation is highly responsive to pathological and physiological conditions, such as aging (Miura and Endo 2016). On the other hand, the evidence is accumulating that glycans might not be mere bystanders but also molecular effectors of aging and age-related diseases (Dall'Olio et al. 2013).

Although it is not the first study to look into the relationship of glycosylation and aging, the seminal paper on IgG glycopattern changes during aging published in 1988 by Parekh et al. (Shade et al. 2015) encouraged a volume of research on plasma/serum and IgG glycosylation during aging. *N*-glycosylation is by far the most common type of glycosylation (Apweiler et al. 1999), methods for *N*-glycan analysis are relatively simple, and plasma/serum is an easily accessible biological sample. The *N*-glycosylation of plasma/serum and IgG, consequently, represents the most explored aspects of human glycosylation in regard to aging, while data on other glycoconjugates is scarce in comparison. For this reason, a vast part of this chapter is focused on total plasma/serum and IgG *N*-glycopattern variations during aging.

Genetic Regulation of Glycosylation Genes that encode proteins directly involved in glycan biosynthesis are abundant, their repertoire of approximately 250–900 genes comprising 1–4% of all human genes, depending on the source (Kikuchi and Narimatsu 2006; Narimatsu 2006; Cummings and Pierce 2014). They encode proteins such as nucleotide-sugar synthases and transporters, glycosyltransferases, glycosidases, lectins, glycan flippases, kinases, mutases, isomerases, proteins that ensure proper folding of glycogene-encoded proteins, and many others (Schachter and Freeze 2009). Genes associated with glycosylation in a broader sense—those coding for a multitude of enzymes, transcription factors, ion channels, and other proteins involved in cell metabolism, endoplasmic reticulum, and Golgi apparatus homeostasis and activity, and secretion—are estimated to correspond to at least 5% of all human genes (Zoldoš et al. 2013; Cummings and Pierce 2014). For a comprehensive review of genes involved in glycosylation, see (Kristic et al. 2021b).

Environmental Regulation of Glycosylation A significant percentage of glycan traits are also associated with lifestyle-related environmental factors, such as diet, smoking, and exercise, as well as with the clinical traits related to the unhealthy state, such as high glucose and cholesterol levels, hypertension, and high body-mass index (Knežević et al. 2009; Menni et al. 2013; Gudelj et al. 2018; Tijardović et al. 2019; Zaytseva et al. 2020).

Glycosylation and aging seem to share the complexity of their respective underlying processes, the intricacy of their regulation, and the plasticity in response to the environment. We will examine the nature of these connections between glycosylation and aging in the following sections.

17.4 Plasma/Serum *N*-Glycome

The analysis of total plasma/serum *N*-glycome generates information about the *N*-glycosylation of plasma/serum glycoproteins. The most abun-

dant of them are immunoglobulins, apolipoproteins, and acute-phase proteins, such as haptoglobin, fibrinogen, alpha-1-antitrypsin, and transferrin (Clerc et al. 2016). Glycosylation changes in many of these proteins are associated with numerous pathological and physiological states (Clerc et al. 2016).

Besides sample accessibility and low volume required for analysis, we owe the plethora of information about plasma/serum *N*-glycans to the high-throughput analytical methods, which allow for the profiling of a vast number of subjects (Knežević et al. 2009; McLachlan et al. 2016; Wittenbecher et al. 2020; Zaytseva et al. 2020). These methods include enzymatic deglycosylation of plasma/serum glycoproteins using PNGase F, and their separation and measurement of their abundance by liquid chromatography (Royle et al. 2008), mass spectrometry (Ruhaak et al. 2008), or capillary electrophoresis (Callewaert et al. 2001). Prior to analysis by liquid chromatography and capillary electrophoresis, and sometimes by mass spectrometry, the released glycans are labeled with fluorescent dyes (Ruhaak et al. 2010b).

N-glycans released from plasma or serum proteins contain up to four antennae, with a variation in terminal monosaccharides (*N*-acetylglucosamine (GlcNAc), galactose or sialic acid), and the presence of bisecting GlcNAc, as well as core and antennary fucose. In addition, glycans containing up to nine mannose residues are present (Fig. 17.1a) (Clerc et al. 2016; Zaytseva et al. 2020).

Given that the main glycoprotein that differentiates plasma from serum is fibrinogen, and that its contribution to total plasma *N*-glycome is minute for most glycan species (Clerc et al. 2016), total serum and plasma *N*-glycomes are usually considered jointly, and we will adhere to this practice here.

17.4.1 Plasma/Serum Glycopattern Dynamics During Aging

17.4.1.1 *N*-Glycosylation

Serum and plasma protein *N*-glycosylation in aging has been extensively researched by several teams (Fig. 17.2, Table 17.1), and will be described here.

Galactosylation Vanhooren and colleagues detected, following desialylation, an increase in agalactosylated (FA2), and a decrease in digalactosylated core-fucosylated biantennary glycan structures (FA2G2) in association with age (Vanhooren et al. 2007, 2008). Consistent discoveries have been made on Croatian and Chinese cohorts, some of which were analyzed without the desialylation step (Knežević et al. 2009, 2010; Ding et al. 2011; Lu et al. 2011). Interestingly, a steeper decline in the abundance of galactosylated core-fucosylated diantennary glycans was observed for females than for males (Knežević et al. 2010; Ding et al. 2011), a fact probably associated with the sharp decline in serum estrogen levels during menopause (Krištić et al. 2014; Ercan et al. 2017). The log of the FA2/FA2G2 ratio after total plasma/serum glycan desialylation was found to gradually increase after the age of 40 years, peaking in >90-year-old subjects (Vanhooren et al. 2010; Catera et al. 2016).

In children, a trend opposite to the one observed in adults occurs—digalactosylated diantennary glycans increase and agalactosylated diantennary glycans decrease with age until puberty (Pučić et al. 2012).

Sialylation The level of total plasma sialic acid (bound and free) increases with age (Mehdi et al. 2012). However, the situation is not as clear regarding sialylated *N*-glycans attached to plasma proteins, the complexity of which decreases with age in children (there is an increase in mono- and disialylated glycans, and a decrease in tri- and tetrasialylated glycans) (Pučić et al. 2012), and increases in people of extreme longevity (discussed later) (Miura et al. 2015), but the data are inconclusive in adults. Most studies did not find significant changes in sialylation during aging (Table 17.1), while mono- and trisialylated glycans have been both positively and negatively correlated with age in two different studies, one of which only found significant changes in males (Knežević et al. 2010; Lu et al. 2011).

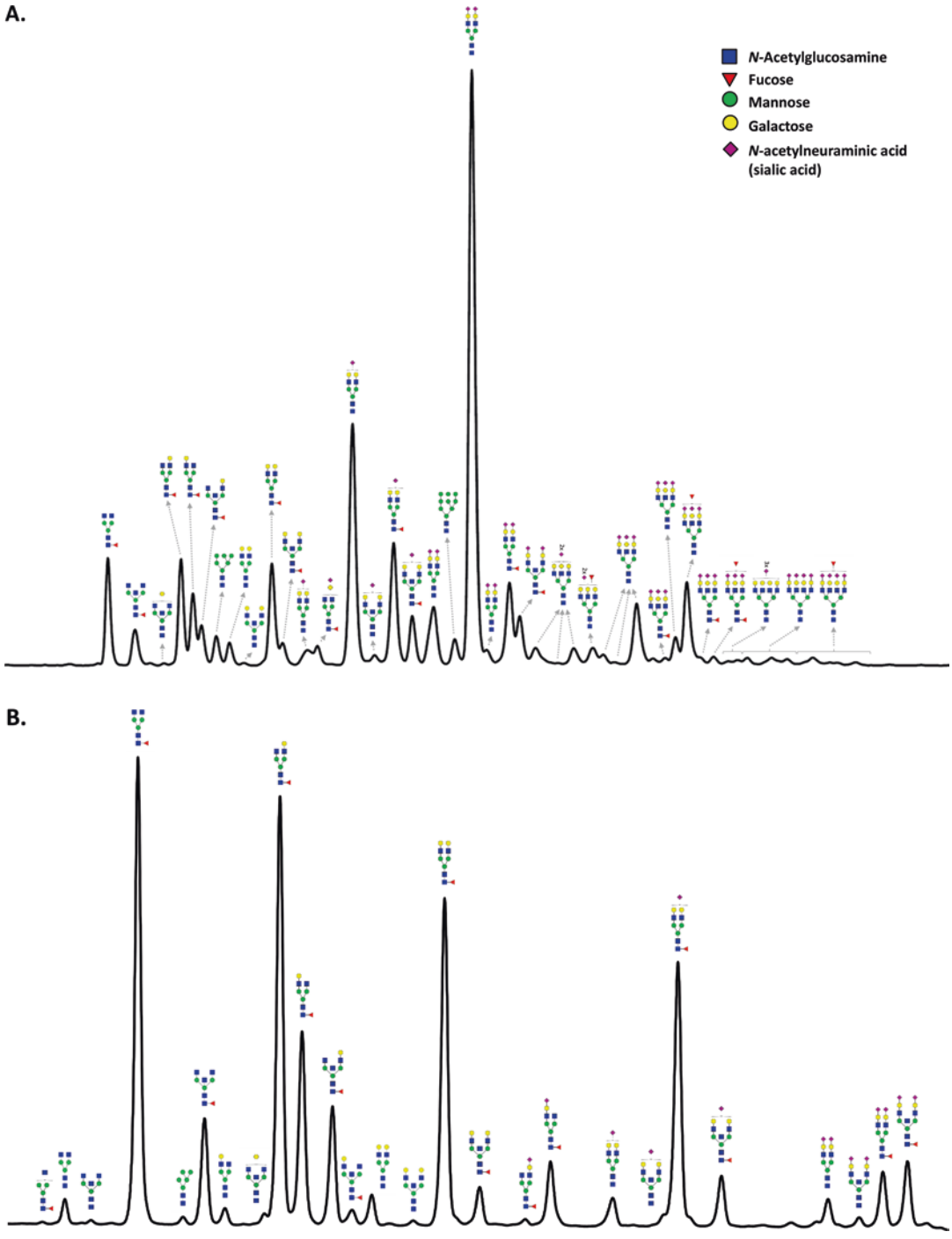

Fig. 17.1 Exemplary ultra-high-performance liquid chromatograms of total plasma *N*-glycome (**a**), and immunoglobulin G *N*-glycome (**b**). The most abundant glycan structures are depicted for each peak

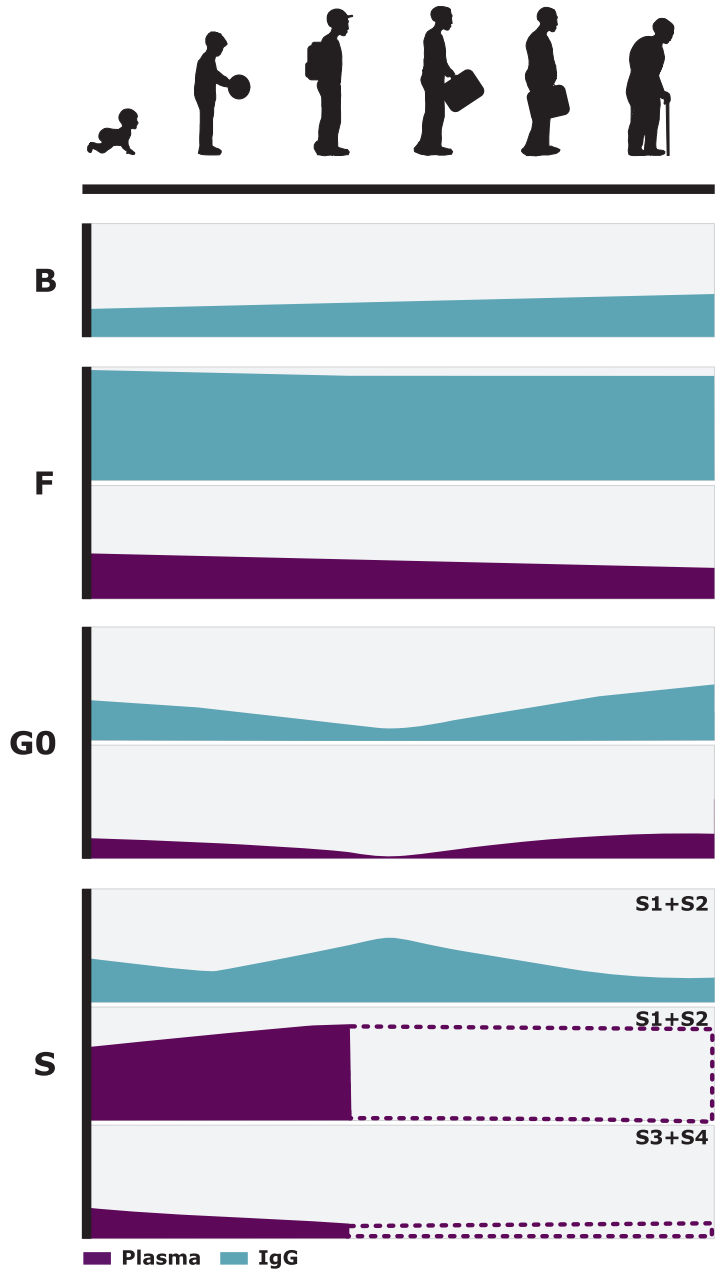

Fig. 17.2 Dynamics of IgG and total plasma *N*-glycome composition during aging. A simplified and symbolic representation for both sexes combined. The y-axes of different glycosylation traits are not comparable and the maximal values do not represent 100%. *G0 = agalactosylated glycans, S1 + S2 = mono- and disialylated glycans, S3 + S4 = tri- and tetrasialylated glycans, B = bisected glycans, F = core-fucosylated glycans. The derived glycosylation traits refer to the terminal monosaccharides of N-glycans. Dashed lines represent unreliable or conflicting data*

Table 17.1 Studies examining the changes in total plasma/serum *N*-glycosylation pattern during aging

Study	Number of subjects	Age range (years)	Analysis type	Glycoanalytical method	Reference
Vanhooren et al. (2007)	221	20–105	Serum	DSA-FACE	Vanhooren et al. (2007)
Knežević et al. (2009)	1008	18–93	Plasma	HPLC	Knežević et al. (2009)
Vanhooren et al. (2009)	338	<32.5 - >100.1	Serum	DSA-FACE	Vanhooren et al. (2009)
Knežević et al. (2010)	1914	18–98	Plasma	HPLC	Knežević et al. (2010)
Vanhooren et al. (2010)	594	3–99	Serum	DSA-FACE	Vanhooren et al. (2010)
Ding et al. (2011)	265	31–60	Serum	DSA-FACE	Ding et al. (2011)
Lu et al. (2011)	212	18–89	Plasma	HPLC	Lu et al. (2011)
Ruhaak et al. (2011)	2396	30–80	Plasma	HPLC	Ruhaak et al. (2011)
Mehdi et al. (2012)	81	21–90	Plasma (+ erythrocyte membranes)[a]	Spectrophotometry	Mehdi et al. (2012)
Pučić et al. (2012)	170	6–18	Plasma	HPLC	Pučić et al. (2012)
Borelli et al. (2015)	79	12–73	Plasma	DSA-FACE, MALDI-TOF-MS	Borelli et al. (2015)
Miura et al. (2015)	16	22–106	Plasma	LC-MS^n	Miura et al. (2015)
Catera et al. (2016)	144	5–105	Plasma	MALDI-TOF-MS	Catera et al. (2016)
Merleev et al. (2020)	97	21–84	Serum	UPLC-ESI-QqQ-MS	Merleev et al. (2020)

[a]Total (bound and free) plasma sialic acid levels – not just that attached to proteins, and not only in *N*-glycans
DSA-FACE = DNA sequencer-assisted, fluorophore-assisted carbohydrate electrophoresis, HPLC = high-performance liquid chromatography, LC-MS^n = liquid chromatography multiple-stage mass spectrometry, MALDI-TOF-MS = matrix-assisted laser desorption/ionization time of flight mass spectrometry, UPLC-ESI-QqQ-MS = ultra-performance liquid chromatography electrospray ionization triple quadrupole mass spectrometry

This ambiguity is not surprising, because the sialylated portion of plasma/serum *N*-glycome comprises of *N*-glycans originating from a plethora of various proteins (Clerc et al. 2016), many of which vary in concentration in response to our physiological and immunological state, such as acute-phase proteins and immunoglobulins.

Core Fucosylation Several studies, performed on both adult and pediatric population, agree that the level of core fucosylation decreases slightly with advancing age (Vanhooren et al. 2007; Knežević et al. 2009; Lu et al. 2011; Ruhaak et al. 2011; Pučić et al. 2012).

Bisection Most studies did not report significant age-related changes in the abundance of plasma *N*-glycans containing bisecting GlcNAc (Table 17.1). However, a lone study reports a marginally increased bisection level in adult males, but not in females (Ding et al. 2011). Another study reports a slight increase in the abundance of a single glycan containing bisecting GlcNAc (FA2B) (Vanhooren et al. 2007).

Extreme Longevity A couple of studies investigated the composition of plasma/serum *N*-glycans in people reaching an extremely old age, looking for traits that differentiate these individuals from the general population. *N*-glycosylation profiling

of plasma proteins in Japanese semisupercentenarians (age mean 106.7 years) showed that they have higher levels of multi-branched and highly sialylated, as well as agalactosylated structures, and lower levels of biantennary structures when compared to aged (age mean 71.6 years) and young (age mean 30.2 years) controls (Miura et al. 2015). While the increase in agalactosylated structures is known to be associated with aging, an increase in complexity (multi-branching, high sialylation) is not implied in aging, but is detected in autoimmune and inflammatory diseases, such as rheumatoid arthritis (Pawłowski et al. 1986), ulcerative colitis, (Miyahara et al. 2013), type 2 diabetes, (Keser et al. 2017; Adua et al. 2019), and chronic pancreatitis (Sarrats et al. 2010). This finding is in accordance with high levels of chronic inflammation detected in semisupercentenarians (with no inflammatory diseases in anamnesis), marked by elevated serum concentration of acute-phase proteins, as well as a triantennary and sialylated haptoglobin glycoform characteristic for inflammatory and age-related diseases (Miura et al. 2015, 2018).

The researchers working on the Leiden Longevity study profiled total plasma *N*-glycomes of more than 2000 subjects, with the aim to evaluate relations between glycans, familial longevity, and healthy aging (Ruhaak et al. 2011). They investigated, among other things, whether genetic predisposition for reaching extremely old age was evident in plasma *N*-glycome composition. They found that two traits containing non-fucosylated biantennary glycans were more abundant in the offspring of nonagenarians (people around 90 years of age) than in age-matched controls (the offspring's partners) (Ruhaak et al. 2011).

In conclusion, the relative composition of plasma *N*-glycome in extremely old individuals in part corresponds to that of the aged, with some characteristics also observed in subjects suffering from inflammatory conditions. The available literature also offers the possibility that the genetic predisposition for longevity is evident in total plasma *N*-glycome, but this remains to be confirmed.

Progeroid Syndromes Progeroid syndromes are a heterogeneous group of rare genetic disorders that, at an early age, mimic some of the clinical and molecular features of physiological aging (Navarro et al. 2006), offering insight into molecular mechanisms of aging. The data on plasma *N*-glycosylation in patients suffering from progeroid syndromes is scarce. Studies performed on subjects with Cockayne syndrome (Vanhooren et al. 2010), Down syndrome (DS) (Borelli et al. 2015), and a single subject with Werner syndrome (Vanhooren et al. 2007), reported plasma glycome changes characteristic for aging (decreased galactosylation level) significantly earlier than the healthy controls. In people with DS some plasma glycome changes specific to DS and not otherwise found in normal aging were also identified, namely a lower level of α-2,6-sialylated tri- and tetragalactosylated *N*-glycan species, which in turn increased with age in DS and were unaffected by aging in controls (Borelli et al. 2015).

17.4.1.2 *O*-Glycosylation

In contrast to *N*-glycosylation, *O*-glycosylation of plasma/serum proteins in aging has, to our knowledge, not been investigated. However, frailty, the most common GS, characterized by a progressive loss of physiological reserves, is accompanied by a major change in the abundance of seven different inflammation-related glycoproteins or their precursors, two of which (fibrinogen and hemopexin) have also shown disease-related changes in *O*-glycosylation (Pacchiarotta et al. 2012; Shamsi et al. 2012; Darvin et al. 2014; Sanda et al. 2016). This suggests there might also be *O*-glycopattern changes associated with aging.

17.4.2 Plasma *N*-Glycosylation as a Biomarker of Healthy Aging

Changes in plasma/serum protein *N*-glycosylation have been reported in several age-related conditions and diseases, such as Alzheimer's

disease, (Chen et al. 2010), Parkinson's disease, (Gotovac et al. 2014) dementia, (Vanhooren et al. 2010) type 2 diabetes, (Testa et al. 2015; Keser et al. 2017; Dotz et al. 2018), colorectal cancer (de Vroome et al. 2018; Doherty et al. 2018), stomach cancer, (Bones et al. 2011) breast cancer, (Saldova et al. 2014) and chronic obstructive pulmonary disease (Pavić et al. 2018). The composition of total plasma/serum *N*-glycome is also proposed to be a biomarker of hepatic health, (Callewaert et al. 2004; Vanderschaeghe et al. 2009, 2010; Capri et al. 2017) and cardiometabolic risk (Wittenbecher et al. 2020).

The results from the Leiden Longevity Study linked an increase in the abundances of certain glycans, such as the afucosylated digalactosylated diantennary structure (A2G2) and afucosylated monogalactosylated monosialylated diantennary structure (A2G1S1), with familial longevity, while a decrease in the level in one of the structures (A2G1S1) was also associated with cardiovascular disease (Ruhaak et al. 2011). This made them conclude that plasma *N*-glycan profile is associated with the degree of healthy aging (Ruhaak et al. 2011). Other studies have found that the abundances of agalactosylated and digalactosylated biantennary core-fucosylated plasma *N*-glycans (FA2 and FA2G2, respectively), are better than chronological age for estimating an individual's biological age and could be used as an aging biomarker (Vanhooren et al. 2010). This establishes the composition of total plasma/serum *N*-glycome as a marker of general health, potentially suitable for prediction and monitoring of disease progression during aging, and assessing the efficacy of anti-aging interventions.

Genetic and Environmental Regulation of Plasma *N*-Glycosylation The composition of human plasma *N*-glycome is highly heritable, the genetic sequence explaining around 60% of the variation for the majority of plasma glycans (Zaytseva et al. 2020). Using a combination of GWASes and high-throughput glycomics, multiple genes were found to influence *N*-glycan levels in human plasma (Lauc et al. 2010; Huffman et al. 2011; Sharapov et al. 2019, 2020). These genes did not overlap with those found through human aging and longevity GWASes in the NHGRI-EBI GWAS catalog at the time of writing this chapter (Buniello et al. 2019).

However, a mouse study has shown a decrease in the lifespan of *Mgat5* (a gene encoding a branching *N*-acetylglucosaminyltransferase) knockout animals (Cheung et al. 2007). These mice are also hypoglycemic, resistant to weight gain on a calorie-rich diet, hypersensitive to fasting, display increased oxidative respiration and reduced fecundity, have fewer muscle satellite cells, less osteogenic activity in bone marrow, and accelerated loss of muscle and bone mass with aging (Cheung et al. 2007). The authors therefore propose that the knockout negatively affected their sensitivity to anabolic cytokines, accumulation of fat stores, tissue renewal, and longevity (Cheung et al. 2007). Interestingly, *MGAT5* was also a hit in several GWASes of human total plasma *N*-glycome, mentioned above (Huffman et al. 2011; Sharapov et al. 2019, 2020). These findings might point to the relevance of the *Mgat5* gene and β-1,6-GlcNAc-branched *N*-glycans for aging and aging-related processes.

The association of the composition of total plasma/serum *N*-glycome and the expression level and activity of glycosyltransferases during aging is ambiguous. Increased expression of the α-1,6-fucosyltransferase (*Fut8*) gene, which codes for the enzyme responsible for the addition of core fucose, in the liver was shown to be strongly linked to the age-related changes in serum *N*-glycosylation in mice (Vanhooren et al. 2011). In humans, the activity of plasmatic β-4-galactosyltransferases (B4GALTs) increased linearly with chronological age, while the activity of plasmatic α-2,6-sialyltransferase 1 (ST6GAL1) showed a quadratic relationship with age, with high levels in children and people above 80 (Catera et al. 2016). The age-adjusted ST6GAL1 activity was positively correlated with the log(FA2/FA2G2) value of desialylated total plasma *N*-glycome, unlike the age-adjusted B4GALTs activity, for which the correlation was

lost after the correction for age (Catera et al. 2016). Since the activity of glycosyltransferases seems to negatively correlate with the products of the enzymatic reactions they catalyze, the interpretation of this finding is elusive. It appears that, in humans, while both the activity of glycosyltransferases, per se, and the plasma *N*-glycopattern are associated with age, plasmatic glycosyltransferases are not directly involved in the glycosylation of plasma *N*-glycoproteins.

The plasma/serum *N*-glycome composition is very plastic in its response to the environment. The factors that affect the plasma/serum *N*-glycosylation range from an unhealthy lifestyle, such as smoking (Knežević et al. 2010), acute sleep deprivation (Chatterton et al. 2020), and exposure to traumatic stress (Moreno-Villanueva et al. 2013), to hormonal status (sex, menopause) (Brinkman-Van Der Linden et al. 1996; Knežević et al. 2010). Plasma/serum *N*-glycome composition associates with the measures reflective of lifestyle and general state of health, such as body-mass index, plasma lipid status, inflammatory markers, and liver enzymes (Knežević et al. 2009, 2010; Reiding et al. 2017; Dotz and Wuhrer 2019).

Feasibility Ease of sampling, and its significant intraindividual stability, both short- and long-term (Gornik et al. 2009; Hennig et al. 2016), allow plasma/serum *N*-glycome to be considered a good biomarker candidate. The recent development of high-throughput methods has enabled rapid analysis of a large number of samples from a small volume (below 10μL) (Royle et al. 2008; Knežević et al. 2011), making total plasma/serum *N*-glycome a suitable biomarker for large epidemiological studies. Its major drawback remains the high inter-individual variability (Knežević et al. 2010; Lauc et al. 2016), requiring longitudinal monitoring for the most informative assessment (Hennig et al. 2016).

In summary, the composition of plasma/serum *N*-glycome is most likely a reflection of physiological processes going on in the body, affected by both genetic background and the environment. There are many different glycoproteins in the plasma/serum, making the interpretation of plasma/serum glycosylation data challenging. It is very hard to determine whether an observed change in glycan levels is caused by a shift in relative protein quantity, relative glycoform quantity, or by a general effect altering the glycosylation of several proteins. Consequently, while the composition of plasma/serum *N*-glycome and aging are strongly correlated, it is very hard to say if the entirety of the plasma/serum *N*-glycome has any functional role in the process of aging. Information about the glycosylation of individual plasma glycoproteins can bring some clarity.

17.5 Immunoglobulin G

IgG is the largest individual contributor to the neutral portion of total plasma and serum glycome (Clerc et al. 2016). However, due to its separate biosynthetic pathway (B cell lineage) and a somewhat distinct *N*-glycome composition, it stands out from the majority of other plasma proteins. Because of this, and because it is the glycoprotein most explored from the aspect of glycopattern dynamics during aging, IgG merits our special attention.

IgG Function IgG is one of the key molecules of our immune system. Its Fab region binds the antigen, while its Fc region attaches to various molecules relevant for downstream effector functions: microbe and toxin neutralization, antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP), and complement activation resulting in complement-dependent cellular cytotoxicity (CDC) and the initiation of inflammation, to mention only the most prominent ones (Bournazos and Ravetch 2017). Moreover, through binding to activating and inhibitory receptors co-expressed on their surface, IgG regulates the activation of innate immune cells (Nimmerjahn and Ravetch 2008). Thus, the duality in IgG structure and, consequently, functionality, allows this versatile molecule to serve as a link between innate and adaptive immunity (Panda and Ding 2015).

IgG Glycosylation Every IgG molecule carries an *N*-glycan attached to the conserved *N*-glycosylation site at Asn 297 on each of its heavy chains (Arnold et al. 2007). Additionally, 15–20% of Fab regions contain *N*-glycans linked to *N*-glycosylation sites that emerge as a result of somatic hypermutation during affinity maturation (Dunn-Walters et al. 2000; van de Bovenkamp et al. 2016). Moreover, 10% of the *O*-glycosylation sites in the extended hinge region of the IgG3 subclass contain core I type (*N*-acetylgalactosamine-galactose) glycans (Plomp et al. 2015). More than 30 distinct *N*-glycan structures have been observed on IgG (Wada et al. 2007), each present at a different proportion of total IgG glycans. This means that, at any given moment, our serum represents a heterogeneous mixture of a large number of differently glycosylated IgG molecules. The most complex IgG *N*-glycan is a digalactosylated and disialylated diantennary structure with core fucose and bisecting GlcNAc (FA2BG2S2) (Fig. 17.1b).

Function of IgG *N*-Glycans IgG glycans help maintain the structure and conformation of the molecule and affect its stability, half-life, and solubility (Mimura et al. 2000; Krapp et al. 2003; Wu et al. 2010; Liu 2015; van de Bovenkamp et al. 2016, 2018). As integral structural parts of the IgG molecule, IgG glycans also affect its effector functions by the modulation of binding to Fc receptors (FcR) on the surface of immune cells. The absence of core fucose increases ADCC by enhanced IgG binding to FcγRIIIA on the surface of natural killer cells (Shields et al. 2002; Shinkawa et al. 2003; Dekkers et al. 2017). Terminal sialic acids act as a switch between IgG pro- and anti-inflammatory phenotype by enhancing IgG binding to type I or type II Fc receptors for asialylated and sialylated IgG, respectively (Pincetic et al. 2014). The functional importance of terminal galactosylation is not straight-forward. By binding to the mannose-binding lectin (MBL), agalactosylated IgG can activate complement through the lectin pathway and thus initiate an inflammatory cascade (Malhotra et al. 1995; Ji et al. 2002; Arnold et al. 2006). Additionally, galactosylated IgG immune complexes suppress the inflammatory cascade through binding to the inhibitory FcγRIIB and the subsequent inhibition of C5a-induced inflammation (Karsten et al. 2012). However, galactosylation was also found to increase CDC through enhanced binding of IgG to C1q, thus emphasizing the complexity of modulation of IgG effector functions by differential glycosylation (Hodoniczky et al. 2005; Peschke et al. 2017). Notwithstanding the seemingly opposing claims, terminal galactosylation is most often considered to direct IgG towards the anti-inflammatory activity.

17.5.1 IgG *N*-Glycopattern Dynamics During Aging

Today the association of IgG glycosylation pattern with aging is an accepted fact (extensively reviewed in (Kristic et al. 2021a)) (Fig. 17.2), supported by the finding that the levels of IgG glycans explain up to 64% of the variation in chronological age (Krištić et al. 2014; Yu et al. 2016). Indeed, the knowledge of the composition of IgG glycome was reported to allow for a prediction of chronological age with an error of ±9.7 years (Krištić et al. 2014).

Galactosylation Galactosylation was the first IgG glycosylation trait for which an age-related change in abundance was reported (Shade et al. 2015). In their pioneering study, Parekh et al. found an association of the level of galactosylated IgG glycans with age (Shade et al. 2015). The galactosylation level was reported to rise from a very young age, reach a peak in early adulthood, and then decrease with the advancement of age (Shade et al. 2015). To this date, galactosylation remains the most investigated IgG glycosylation trait in respect to aging, both on the level of released total (Fab + Fc) glycans, and on the level of IgG glycopeptides, which offer information about subclass-specific Fc glycosylation.

The inter-individual variability in IgG glycome composition is quite large, particularly

regarding the level of galactosylation (Pučić et al. 2011; Gornik et al. 2012). Therefore, large sample sets are required for a reliable estimation of glycopattern change during aging when a cross-sectional study design is applied. However, mostly due to ethical constraints, pediatric populations were chiefly studied on smaller sample numbers (up to 164) (Kristic et al. 2021a), with the largest study covering over 600 children (Pezer et al. 2016). The results coming from studies on children are not simple to interpret. Several studies report an increasing proportion of digalactosylated and a decreasing proportion of agalactosylated IgG glycans with age (Chen et al. 2012; Shade et al. 2015; Cheng et al. 2020). However, in other studies, this trend was observed either in single sex (Yamada et al. 1997; Pučić et al. 2012), a single subclass (IgG4), (Pezer et al. 2016) or not at all (De Haan et al. 2016). The inconsistency between results of studies performed on pediatric populations likely originates in smaller sample sets (compared to adult populations) and/or study design (Kristic et al. 2021a).

In contrast to the studies performed on children, studies performed on adult populations mainly agree on general conclusions: early adulthood is marked by a similar level of agalactosylated and digalactosylated IgG glycans. However, while the abundance of agalactosylated glycans increases, the abundance of digalactosylated glycans decreases with age. This is true both on the level of released (Fab + Fc) glycans and on the level of subclass specific Fc glycans (Tsuchiya et al. 1993; Keusch et al. 1996; Yamada et al. 1997; Shikata et al. 1998; Selman et al. 2010; De Haan et al. 2016). Large-scale multi-population studies consisting of up to several thousand subjects (the largest study comprising 5117 subjects (Krištić et al. 2014)) confirmed these findings (Ruhaak et al. 2010a; Pučić et al. 2011; Chen et al. 2012; Baković et al. 2013; Krištić et al. 2014; Yu et al. 2016; Plomp et al. 2017). The results of studies examining the association of the level of monogalactosylated IgG glycans with age are inconsistent (Kristic et al. 2021a).

Interestingly, when it comes to galactosylation, a discrepancy between the two sexes was discovered for the adult population (Krištić et al. 2014). In males, the galactosylation level declines at a steady pace with advancing age (Krištić et al. 2014). Females start with galactosylation levels on average higher than in men, which gradually decrease with age (Krištić et al. 2014). After this, the galactosylation level continues to decrease at the initial steady pace, but the overall level of galactosylation is lower than in men (Krištić et al. 2014). This finding suggests the galactosylation level is associated with the hormonal status. The hypothesis was confirmed soon after, in a rare human interventional study, where estrogen supplementation prevented the drop in IgG galactosylation caused by the hormonal induction of menopause (Ercan et al. 2017).

Sialylation Since the level of sialylation (particularly disialylation) is significantly higher among Fab compared to Fc glycans (van de Bovenkamp et al. 2016), the conclusions on sialylation dynamics during aging are inevitably influenced by the analytical method applied.

The studies on pediatric populations, mostly analyzing the Fc glycans, observed a decrease of sialylated glycans until prepuberty (about 10 years of age), after which the trend probably reverses, accompanying the abundance dynamics of galactosylated glycans (Chen et al. 2012; De Haan et al. 2016; Pezer et al. 2016; Cheng et al. 2020). A single study that analyzed released (Fab + Fc) glycans found no age-related difference in total IgG sialylation level (Pučić et al. 2012), indicating that the age-related change is driven by the change in the abundances of Fc-linked glycans.

Most large-scale studies on the adult population report a decrease in the abundance of sialylated IgG glycans after early adulthood, and sex-specific dynamics similar to the one observed for galactosylation (Pučić et al. 2011; Chen et al. 2012; Baković et al. 2013; Krištić et al. 2014; Yu et al. 2016; Plomp et al. 2017). Since the galactosylated structures act as a substrate for sialyltransferases (Rini and Esko 2015), it is no surprise that in healthy populations the change in the

sialylation level associates, to a degree, with the change in the galactosylation level.

Bisection The abundance of IgG glycans containing bisecting GlcNAc was mostly reported to increase during childhood and adolescence (Yamada et al. 1997; Chen et al. 2012), as well as during adult life (Yamada et al. 1997; Shikata et al. 1998; Selman et al. 2010; Pučić et al. 2011; Chen et al. 2012; Baković et al. 2013).

Core Fucosylation Studies performed on pediatric populations report that increasing age is associated with a decrease in the abundance of core fucosylated IgG (Chen et al. 2012; De Haan et al. 2016; Pezer et al. 2016; Cheng et al. 2020). The results for adult populations are conflicting. However, even in cases where a change in the abundance is reported, its extent is always mild (Kristic et al. 2021a).

The origins of changes in IgG glycosylation profile that occur during aging are unclear. Some of the proposed mechanisms include changes in expression and/or activity of glycosyltransferases and glycosidases, production and/or localization of activated nucleotide sugar donors, and unequal turnover rate of differentially glycosylated IgG (Kristic et al. 2021a).

17.5.2 Inflammaging

Inflammaging refers to the pro-inflammatory reshaping of the immune system characteristic for the elderly, which is presented as sustained chronic asymptomatic low-grade inflammation (De Martinis et al. 2005; Pinti et al. 2016; Monti et al. 2017). It is likely caused by long-term exposure to various endogenous and exogenous inflammatory stimuli, whose origins vary from different microorganisms and toxins that we encounter to inflammatory cytokines secreted by senescent cells, and the presence of necrotic and damaged cells (Dall'Olio 2018). Attenuation of inflammation is indeed a good predictor of healthy aging (Arai et al. 2015), thus establishing the association of inflammaging with the risk of age-related diseases (Franceschi 2007; Pinti et al. 2016).

The decrease in the level of galactosylation and sialylation, accompanied by an increase in the level of bisection, suggests that our IgG glycopattern gradually becomes more pro-inflammatory as we age. This phenomenon is thought to contribute to the age-related imbalance between the pro-inflammatory and anti-inflammatory networks, whose original purpose is to help us fight pathogens and maintain homeostasis, respectively (Franceschi et al. 2007). Due to the potential of IgG glycans to modulate the molecule's inflammatory capacity and the general threshold of immune activation, it is proposed that the age-related gradual accumulation of agalactosylated IgG is not only one of the hallmarks of aging, but also a contributor to its exacerbation (Fig. 17.3) (Franceschi et al. 2000; De Martinis et al. 2005; Monti et al. 2017). Thus, in a self-amplifying loop, agalactosylated IgG acts as both —a "by-product" of aging and a mechanistic effector of its pathogenic changes (Franceschi et al. 2007; Dall'Olio et al. 2013).

17.5.3 IgG *N*-Glycosylation as a Biomarker of Biological Age

The changes in IgG glycopattern that occur during aging also characterize many various diseases, namely inflammatory, autoimmune, and malign diseases, some of which are age-related (Lauc 2016; Gudelj et al. 2018). The most typical of these diseases include rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, Alzheimer's disease, and several types of cancer (Lauc 2016; Gudelj et al. 2018). While it is likely that the characteristic changes in IgG glycoprofile reflect common underlying inflammatory processes, the precise mechanisms that bring these changes about currently represent one of the biggest and most important unknowns of IgG glycosylation. Shedding light on the regulation and con-

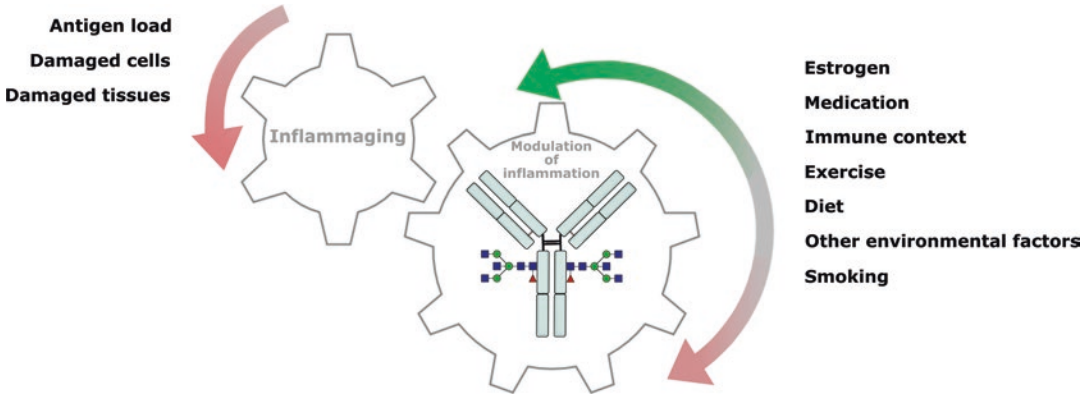

Fig. 17.3 In a self-amplifying vicious loop, agalactosylated immunoglobulin G glycans are suggested to be both, a byproduct and an effector of aging. Whether lifestyle changes and other environmental interventions can decrease the rate of aging through a directed modification of immunoglobulin G glycopattern, remains to be seen

sequences of IgG glycopattern dynamics during aging could provide new insights into the basic biological mechanisms underlying aging and age-related diseases.

Genetic and Environmental Regulation of IgG *N*-Glycosylation The composition of IgG glycome is heavily influenced by genes and thus highly heritable (Pučić et al. 2011; Menni et al. 2013), various genes associated with IgG *N*-glycome being pleiotropic with different autoimmune and inflammatory diseases and hematological cancers (Lauc et al. 2013; Shen et al. 2017; Wahl et al. 2018b; Klarić et al. 2020). None of these genes were found to be associated with aging and longevity according to the NHGRI-EBI GWAS register searched at the time of writing this chapter (Buniello et al. 2019).

IgG *N*-glycome composition is also associated with lifestyle factors, such as smoking, fitness, diet, and exposure to stress (Pavić et al. 2018; Wahl et al. 2018a; Konjevod et al. 2019; Russell et al. 2019a), as well as with clinical and phenotypical variables related to unhealthy lifestyle and poor immune and cardiometabolic health, such as serum glucose and insulin levels, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, alanine aminotransferase, aspartate aminotransferase, C reactive protein, waist circumference, body-mass index, waist-to-height ratio (Krištić et al. 2014; Plomp et al. 2017; Gudelj et al. 2018; Russell et al. 2019b).

Positioned at the crossroads between genetic make-up, environment (Menni et al. 2013), immune response, and aging (Dall'Olio et al. 2013), IgG glycans appear to have the potential to distinguish between healthy and unhealthy aging, as evidenced by the association of IgG glycopattern with general immune activation (De Jong et al. 2016), and expected lifespan (Štambuk et al. 2020). We, therefore, propose that IgG glycoprofile can be used as an assessment of a general state of health, that is, a biomarker of biological age (Vilaj et al. 2019). Likewise, a recent multi-omics graphical model demonstrating the interconnectivity of age-related diseases highlights IgG glycome composition as a molecular marker of aging that might drive disease comorbidities (Zierer et al. 2016).

The research efforts are currently focused on longitudinal studies aiming at changing the individuals' IgG glycosylation pattern for the improvement of biological age. Several intervention studies succeeded in modifying the IgG glycopattern by introducing lifestyle changes, such as exercise-induced weight loss, repeated sprint training, the introduction of dietary supplements, estrogen supplementation, and extensive weight loss after bariatric surgery (Ercan et al. 2017; Sarin et al. 2019; Tijardović et al. 2019; Peng et al. 2019; Jurić et al. 2020; Greto et al. 2020).

These results offer a glimmer of hope that one day we might be able to reverse the rate of biological aging. IgG glycosylation analysis will possibly be one of the biomarkers used to evaluate the success of interventions aimed at this purpose.

Feasibility IgG *N*-glycome shares the main feasibility features with plasma/serum total *N*-glycome. Its accessibility, intraindividual stability, the existence of affordable high-throughput methods, and small blood volume necessary for the analysis make it a suitable biomarker. The high interindividual variability can be bypassed by longitudinal monitoring, similar to plasma *N*-glycome, or by comparison with age- and sex-matched baseline values from subjects of the same ethnicity.

In summary, IgG glycopattern changes with advancing age, both in childhood and in adulthood. The adult period is characterized by a decrease in the level of terminal galactosylation and sialylation, and an increase in the level of bisection. Influenced by both genes and the environment, IgG glycans can modulate IgG effector functions and its inflammatory capacity, making them a likely contributor to the aging process on the molecular level, as proposed by the inflammaging hypothesis. Many inflammatory, autoimmune and neoplastic diseases share the glycosylation profile of serum IgG that characterizes aging, probably due to a common underlying inflammatory component of disease pathology. This confirms IgG is a good read-out of the general state of health or biological age. First intervention studies that resulted in modulation of IgG glycoprofile have opened the door to the idea that directed alteration of IgG glycome composition might once allow us to prevent or revert the age-related conditions.

17.6 Animal Models for Serum and IgG *N*-Glycosylation

Even though results obtained from model animals cannot be directly translated to humans, the use of animal models is necessary for biomedical research. Since they allow for various treatments and interventions on an organismal level, they often provide useful information that could not be attained otherwise. Furthermore, animal models provide a broader picture, revealing if the conclusions obtained on human biological processes are specific to humans, or if they are a general trait of multiple species. All of this is true for the application of animal models in the research of aging.

Mouse Serum *N*-Glycosylation A study on healthy C57BL/6 mice found significant age-related changes in serum glycosylation of animals up to 23 months of age (Vanhooren et al. 2011). Most notably, the abundance of agalactosylated core-fucosylated diantennary glycans (FA2) increased with age, which is in accordance with human studies (Vanhooren et al. 2011). An increase in the abundance of digalactosylated core-fucosylated biantennary glycans (FA2G2) and a decrease in digalactosylated afucosylated biantennary glycans (A2G2) was also reported, but since these results refer to the glycosylation profile after desialylation, it is impossible to draw any conclusions on galactosylation or sialylation of biantennary glycans separately (Vanhooren et al. 2011). The same trends were true for the antibody-depleted serum, pointing to the influence of liver-originating glycoproteins in the total serum *N*-glycome in mice (Vanhooren et al. 2011). A similar, but accelerated trend was noticed in mice with a genetically shortened lifespan, while the opposite trend was observed in mice fed a calorically restricted diet (Vanhooren et al. 2011). Thus, on the level of total serum *N*-glycome, "healthy" diet is associated with slower aging in mice. Additionally, in the same study, an age-related increase in core-fucosylation of total serum proteins was observed, in association with an increased expression and activity of α-1,6-fucosyltransferase (Fut8) in the liver. This finding suggests that the glycosylation machinery in mouse liver cells is affected during aging (Vanhooren et al. 2011). In vitro experiments on primary mouse hepatocytes suggest *Fut8* expression might be functionally involved in the aging process through the modulation of insulin-like

growth factor 1 (IGF-1) signaling pathway (Vanhooren et al. 2011).

Mouse IgG *N*-Glycosylation A recent study that explored Fc glycosylation in BALB/c mice from birth to adulthood (5–60 days of age) reported a significant decrease in the levels of agalactosylated and monogalactosylated structures, combined with an increase in the level of sialylated and α-1,3-galactosylated (α-Gal) structures (Barrientos et al. 2020). This pattern of change, observed in all IgG subclasses, indicates that the transition between neonatal life and adulthood in mice is accompanied by a reduction of inflammatory IgG antibodies (Barrientos et al. 2020).

A study on adult mice (2–8 months of age) showed an age-dependent increase of agalactosylated IgG for six out of seven analyzed strains (Bodman et al. 1994).

The most comprehensive study on IgG glycosylation during mouse aging examined C57BL/6 mice of both sexes in 12 time points between 6 and 80 weeks of age, covering a period from puberty to old age (Han et al. 2020). In the period between puberty (6 weeks) and mature adulthood (12–16 weeks), the level of IgG galactosylation remained constant, the level of core fucosylation increased with age, while the levels of bisection, sialylation, α-1,3-galactosylation, high-mannose glycans, and monoantennary glycans decreased with age (Han et al. 2020). Specifically, the abundance of the FA2 structure increased until 16 weeks (Han et al. 2020), confirming the previous finding (Bodman et al. 1994). In the period from mature adulthood (12–16 weeks) to old age (80 weeks), the level of IgG galactosylation increased, the levels of high-mannose glycans and monoantennary glycans increased until 40 weeks and subsequently decreased, the level of bisection and core fucosylation remained stable until an increase at 80 weeks, while the level of sialylation increased steadily and then dropped sharply at 80 weeks of age (Han et al. 2020). The most pronounced age-related change was the decrease in the abundance of core fucosylated monogalactosylated monosialylated diantennary glycans (FA2G1S1) (Han et al. 2020). The final finding replicated what we had observed earlier on multiple strains in our study aimed at the investigation of genetic regulation of IgG glycosylation (Krištić et al. 2018). The detailed study by Han et al. indicates that mouse might not be a suitable model for human IgG glycosylation in aging research, as there is hardly any agreement between IgG glycopattern dynamics during mouse and human aging. In particular, the two most prominent IgG glycosylation traits (galactosylation and sialylation) seem to follow the opposite trends in the two organisms. More in-depth research is required to confirm the initial findings.

Importantly, the stark differences in the level of IgG galactosylation and sialylation between different mouse strains of the same age (Bodman et al. 1994; de Haan et al. 2017; Krištić et al. 2018) emphasize the need for the selection of appropriate strain when conduction aging and intervention mouse studies that involve IgG glycosylation (Mitchell et al. 2015).

Rat Serum Glycosylation Research on healthy rats showed that, similar to humans (Mehdi et al. 2012), total (bound and free) serum sialic acid increases as a function of old age, and is probably a combined result of several causes, such as the increased expression of acute-phase proteins, internal organ damage, and oxidative damage of erythrocytes (Kumar and Rizvi 2013). Treatment of young and old Wistar rats with a glycolytic inhibitor 2-deoxy-D-glucose (2-DG), a caloric restriction mimetic, showed an increase in reactive oxygen species (ROS), followed by the augmentation of ROS defense mechanisms and a decrease in total plasma sialic acid content, suggesting that 2-DG treatment could be used as an intervention strategy aimed at slowing down the rate of aging (Saraswat et al. 2019).

To our knowledge, there are no studies on rats that examined the dynamics of their plasma/serum or immunoglobulin *N*-glycosylation during aging.

17.7 Glycosylation of Other Proteins, Body Fluids, Cells, and Tissues

17.7.1 Other Serum Proteins

The age-related glycosylation dynamics have mostly been studied on the *N*-glycome of total serum or plasma proteins and IgG. However, there are still reports, albeit scarce, on the age-related alterations in the glycosylation of other proteins. Individual proteins that have been glycoprofiled in regard to aging include acute-phase proteins important in inflammation, such as fibrinogen, (Catera et al. 2016; Gligorijević et al. 2018) transferrin, (Bergström and Helander 2008; Dunston et al. 2012) alpha-2-macroglobulin, (Calvert et al. 2019; Šunderić et al. 2019); as well as thrombospondin-1 and immunoglobulin M (Catera et al. 2016). In general, these changes are either slight or not fully elucidated, mostly due to small amounts of samples and the complexity of the analysis.

However, analysis of samples from the Leiden Longevity Study showed that the glycosylation pattern of the α1-antitrypsin-enriched fraction is associated with age (Ruhaak et al. 2013). Most of the glycans that are negatively correlated with age are afucosylated sialylated di- and triantennary glycans, while a fucosylated disialylated triantennary glycan (FA3G3S2) is positively correlated with age (Ruhaak et al. 2013). In the same study, only the core-fucosylated monogalactosylated bisected glycan (FA2BG1) from the immunoglobulin A-enriched fraction showed an age-associated increase in abundance (Ruhaak et al. 2013).

17.7.2 Other Body Fluids

Saliva Saliva protein content significantly overlaps with that of plasma (Loo et al. 2010). By contrast, its collection is easier, less invasive, and more cost-effective (Malamud 2011). Glycoproteins comprise an important part of total salivary proteins, and there have been several studies focused on the age-associated changes in their abundance and glycosylation profile (Xu et al. 2019).

The concentration of numerous human salivary glycoproteins, many of which are involved in the innate immunity against microorganisms (Shinozaki et al. 2001; Simantov et al. 2001; Bingle and Craven 2002; Hofman et al. 2007), increases with age (Sun et al. 2014). This holds true for mucins - heavily glycosylated proteins with important functions in defense against microbial infections, and a sialylated mucin-like molecule in mice (Sonesson et al. 2008; Iida et al. 2019). In mice, this increase was associated with a rise in the expression of two submandibular gland sialyltransferases, most probably to keep up with the increased concentration of their protein substrate (Iida et al. 2019). Though the antimicrobial role of mucins suggests the increase in their levels is beneficial, their presence makes saliva more viscous and can lead to xerostomia or dry mouth, a common age-related condition that can cause a drastic decline in oral health (Stack and Papas 2001).

Components of human saliva that contain sialic acid can inhibit influenza A infectivity by the inhibition of viral hemagglutination activity and agglutination of virions in vitro (Hartshorn et al. 2003; White et al. 2009). Interestingly, in humans, the majority of anti-influenza salivary activity is acquired over the first year of age, the phenomenon also occurring in infants with no prior exposure to the virus (Gilbertson et al. 2019). The anti-viral effect is strongest in the elderly, partly because of a higher content of terminal sialic acid residues in their saliva (Qin et al. 2013). This suggests that glycans, namely sialic acids, are an important factor in the protection from influenza infection rendered by the saliva; and that the modification of the composition of salivary glycans in the elderly might occur in response to the higher risk of infectious diseases with advancing age (Decker 2010). In particular, sialic acids on salivary mucins and several other glycoproteins (namely surfactant protein A, glycoprotein-340, pentraxin-3, and α-2-macroglobulin) are thought to act as decoy

ligands for viral hemagglutinin, and the change in salivary glycome composition might be attributed to the age-related changes in their concentrations (White et al. 2009).

Urine Much like saliva, urine can be sampled in a non-invasive, cost-effective manner. Of note, the high salt content and the wide pH range of urine samples might present a challenge for protein isolation and glycan analysis. Urine contains many glycosylated entities: glycoproteins, such as uromodulin, vasorin, and glycophorin-C (Halim et al. 2012); and glycosaminoglycans (GAGs), such as dermatan sulfate, keratan sulfate, chondroitin sulfate, and heparan sulfate (Coutinho et al. 2012). These compounds have been the focus of potential biomarker discovery in multiple studies (Harpole et al. 2016).

GAGs are important for inter-cell and cell-matrix interactions and cellular migration, which are intense during development (Lindahl et al. 2015). The increased turnover of GAGs due to the remodeling of the extracellular matrix in the early stages of life results in their heightened excretion in the urine (Ohkawa et al. 1972; Komosinska-Vassev et al. 2014). Conversely, both of these processes abate during healthy aging (Ohkawa et al. 1972; Komosinska-Vassev et al. 2014).

There are significant differences between adult and pediatric *N*-glycosylation profiles of urine proteins: children exhibit a higher level of high-mannose, asialylated afucosylated glycans, neutral fucosylated, and agalactosylated glycans, and a lower level of trisialylated glycans compared to adults (Li et al. 2020). The relevance of these differences is unknown.

In conclusion, much less is known about saliva and urine alteration in glycosylation during aging compared to plasma/serum. The changes observed in saliva glycan content probably have a functional role, especially in protection against pathogens. The glycoconjugates in urine are a result of metabolic turnover and therefore probably not functionally relevant, but offer an insight into metabolic changes accompanying aging. Since these biological samples are easily accessible, more research on this topic is warranted to fully assess their potential as biomarkers of healthy aging.

17.7.3 Cells and Tissues

Glycans on the cell surface are crucial for cellular interactions. They are the first to come into contact with the entire surrounding of a cell, from other cells and connective tissues to pathogens. Since the interactivity of cells changes with age, it is no surprise that aging is also accompanied by a change in the composition of cell surface glycans.

Erythrocytes Glycans attached to human erythrocytes are known to contain large amounts of sialic acid, which are major contributors to the erythrocyte net negative surface charge (Eylar et al. 1962; Fernandes et al. 2011). The level of erythrocyte-bound sialic acid decreases with age (Gattegno et al. 1976; Mazzanti et al. 1997; Mehdi et al. 2012), and is markedly lower in different age-related diseases such as cardiovascular diseases (Hadengue et al. 1998; Nigam et al. 2006) and blood cancers (Aminoff et al. 1980). In some age-related diseases, however, the opposite is true: patients suffering from type 2 diabetes (Mazzanti et al. 1997) and gastric cancer (Sun 1988; Arivazhagan et al. 1998) exhibit higher levels of erythrocyte-bound sialic acid than healthy controls. In vitro enzymatic removal of sialic acid residues by neuraminidase resulted in the loss of the net negative charge in human, rat, and rabbit erythrocytes (Durocher et al. 1975). Additionally, desialylated erythrocytes were rapidly cleared by the liver in rats and rabbits, suggesting that aging is also a cause for the decrease in red cell lifespan, presumably through the sialic acid loss caused by oxidative stress (Durocher et al. 1975). The age-related sialic acid loss from erythrocyte membranes is also suggested to play a role in the increased risk for the adhesion of erythrocytes to the vascular wall, coupled with an increased risk for cardiovascular diseases (Klei et al. 2018). These findings position the membrane content of

sialic acid as a marker of cellular senescence in erythrocytes.

Another important group of glycans bound to erythrocytes is the one defining ABO blood groups, which consists of A, B, and H antigens. A, B, and AB blood groups are determined by additional glycans attached to the H antigen, while the O blood group only consists of the H antigen without any additional structures (Stanley and Cummings 2015). Although the importance of blood groups is obvious in some instances (e.g., blood transfusion), it might come as a surprise that they are possibly also linked with lifespan: blood type B has been proposed to indicate exceptional longevity, since it is more frequent in centenarians than in the general population (Shimizu et al. 2004).

Fibroblasts Studies on human fibroblast cultures showed that decreased levels of α-2,3-and α-2,6-sialylated *N*-glycans, and α-2,3-sialylated *O*-glycans on cell surface proteins are associated with aging and cellular senescence, respectively (Itakura et al. 2016, 2018). This decrease in cell surface sialic acid levels was possibly caused by the detected decrease in ST6GAL1 activity in aging fibroblasts (Tadokoro et al. 2006), and an increase in the expression of a cell membrane sialidase (Sasaki et al. 2017). Interestingly, it was also linked to the intracellular accumulation of α-2,6-sialylated glycoproteins, as determined by lectin microarray (Itakura et al. 2018). This finding suggests that a balance between intracellular and membrane glycoproteins is important for proper cell functioning, though the precise mechanism through which the two are linked remains to be elucidated (Itakura et al. 2016, 2018).

Brain Aside from single cells, aging-related glycosylation changes have been reported for whole tissues. For example, brain glycosylation is known to be region- and age-specific in rats, where the levels of striatum heparan sulfate and the overall levels of terminally galactosylated *N*-glycans decrease with age;(Raghunathan et al. 2018) and mice, where the abundance of sialic acid and immature mannose-containing glycoproteins increase with age (Simon et al. 2019). Data acquired from human brain samples revealed the presence of significant changes in the abundance of *N*- and *O*-glycopeptides (a decrease in tri- and tetraantennary sialylated *N*-glycopeptides and an increase in biantennary sialylated, high-mannose *N*-glycopeptides and *O*-glycopeptides), (Brunngraber and Webster 1986), changes in prefrontal cortex heparan sulfate structure (Raghunathan et al. 2020), and total brain glycome composition related to advancing age (Lee et al. 2020). However, due to the complexity of analysis and sample inaccessibility, the research on human brain glycosylation during aging is scarce and a comprehensive study using state of the art methodology is lacking.

Muscles Like neural tissue, muscles are known to deteriorate with age, which results in sarcopenia, one of the most common GSs (Cruz-Jentoft et al. 2010). Accelerated muscle aging and lowered performance have been correlated with decreased sialic acid content in the muscle tissue of mice deficient in the gene for UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), an enzyme crucial for sialic acid biosynthesis (Hanisch et al. 2013). Heterozygous $Gne^{+/-}$ mice showed a significantly lower running performance and a significantly reduced level of sialic acid in muscle tissue compared to wild type ($Gne^{+/+}$) mice, indicating the importance of glycosylation in proper muscular function (Hanisch et al. 2013).

Placenta A study that investigated *N*-glycans of cell membrane proteins from the human placenta showed that these proteins contain more α-2,3 sialic acid, and less α-2,6 sialic acid and core fucose in older women (Robajac et al. 2014). The relevance of this finding is unknown.

17.8 Enzymes Involved in Glycosylation

β-Galactosidase β-Galactosidase is expressed by senescent, but not quiescent or terminally differentiated fibroblasts and keratinocytes in cul-

ture (Dimri et al. 1995). In addition, the expression of the enzyme in the same cell types in skin samples from donors of different age is age-dependent (Dimri et al. 1995). Increased intracellular β-galactosidase activity is routinely used as a biomarker of senescent cells (Dimri et al. 1995; Maier et al. 2007) and is possibly also related to the age-dependent decrease in galactosylation level observed on IgG.

α-1,6-Fucosyltransferase 8 The putative relevance of FUT8 in the aging process has already been mentioned. *Fut8* expression is almost nonexistent in adult mice and increases slowly during aging (Vanhooren et al. 2011). Interestingly, FUT8 seems to play a crucial role during postnatal development as well, since *Fut8* knockout mice die soon after birth (Wang et al. 2006), and the expression of *Fut8* is high in mice up to 8 weeks of age (Vanhooren et al. 2011).

Plasmatic Glycosyltransferases Even though the importance of intracellular glycosyltransferases on cellular glycome composition is long-established, it has only recently been discovered that glycosylation is not an exclusively intracellular process. It was previously thought that the concentration of glycosyltransferases and their substrates was only sufficient to fuel the glycosylation reaction inside of cells, but research on platelets and IgG glycosylation proved otherwise, especially in inflammatory conditions (Nasirikenari et al. 2006, 2010, 2014; Wandall et al. 2012; Jones et al. 2016; Manhardt et al. 2017). However, recent research has shown that plasmatic ST6GAL1 does not have a major influence on IgG glycosylation in steady-state (Schaffert et al. 2020). As already mentioned, the activity of plasmatic B4GALTs increases with age, and that of ST6GAL1 is the highest in children, and adults above the age of 80 (Catera et al. 2016). Plasmatic β-galactosidase activity also appears to increase with age, consistent with its heightened activity in senescent cells, but unrelated to the age-related increase of agalactosylated IgG glycans (Spazzafumo et al. 2017).

The level of plasmatic glycosyltransferases might, however, be relevant for plasmatic *N*-glycoproteins other than IgG. For instance, elevated plasmatic ST6GAL1 in the offspring of centenarians, compared to the offspring of non-long-lived parents (Dall'Olio 2018) is consistent with high sialylation level of total plasma *N*-glycoproteins in semisupercentenarians (Miura et al. 2015), but the relevance of this finding, aside from hypothesizing on the regulation of inflammatory potential, remains to be investigated.

Cancer Cell Enzymes In contrast to the low cell-surface sialylation of aging cells, a heightened level of cell-surface sialic acid is a universal and the most prominent trait of aberrant glycosylation in cancer cells (Dragsten et al. 1980; Pinho and Reis 2015), agreeing with the opposing nature of these two biological phenomena—cellular aging and immortalization (Ukraintseva and Yashin 2003). The most notable of cancer-associated sialyltransferases is ST6GAL1, important for all phases of tumor development and progression (Garnham et al. 2019). The complex mechanisms through which hypersialylation is beneficial for cancer cells include the modulation of affinity for sialic-acid binding lectins on immune cells, which contributes to tumor immune evasion and cancer-associated inflammation (Pearce and Läubli 2015). An elevated level of surface-bound sialic acid, coupled with heightened sialyltransferase activity, is also observed in metastatic cells (Dobrossy et al. 1981). Moreover, cell-surface sialic acid is considered a therapeutic target: overexpression of sialidases and subsequent desialylation of tumor cells resulted in suppression of tumor growth, survival, and metastasis in human and mouse cell cultures, as well as upon injection of desialylated cancer cells into mice (Kato et al. 2001; Sawada et al. 2002; Uemura et al. 2009).

Aberrant expression of all four human sialidases (neuraminidase 1, 2, 3, and 4) has been observed in tumorigenesis and/or cancer progression (Haxho et al. 2016). Unlike the aforementioned enzymes, these sialidases do not seem to affect the surface of the cancer cells, acting

instead on intrinsic tumorigenic pathways (Haxho et al. 2016). For example, neuraminidase 1 regulates cancer growth through putative involvement in several pathways, modifying the activity of multiple TOLL-like receptors and receptor tyrosine kinases (Haxho et al. 2016). Its inhibition had a therapeutic effect in both human cell lines and murine tumor models (Haxho et al. 2014; O'Shea et al. 2014; Qorri et al. 2020).

Additionally, various glycosidases are known to be secreted by tumor cells into surrounding tissue, their heightened activity enabling metastasis through degradation of the extracellular matrix (Bernacki et al. 1985).

Enzymes Involved in Glycosaminoglycan Synthesis One of the causes of the progeroid type of the Ehlers-Danlos syndrome, a rare genetic disorder of the connective tissue, is suggested to be a defect in a galactosyltransferase involved in the synthesis of GAGs (Quentin et al. 1990). People suffering from this condition are unable to form a functional linkage crucial for the formation of several proteoglycans (heparin sulfate and chondroitin/dermatan sulfate) that play important roles in tissue formation and development (Okajima et al. 1999).

17.9 Siglecs

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are lectins expressed on the surface of immune cells, largely acting as inhibitory receptors through their cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) (MacAuley et al. 2014). Siglecs' main role is dampening the immune and inflammatory processes through their interaction with terminal sialic acids on the surface of innate and adaptive immune cells (MacAuley et al. 2014). Their expression level and activity are positively associated with longevity in mammals (Schwarz et al. 2015), probably because of their ability to restrain the age-related chronic increase in inflammation and prevent autoimmunity through down-regulation of B-cell-mediated immune responses (Müller et al. 2015). It is, therefore, a plausible proposition that Siglecs participate in the regulation of the aging process (MacAuley et al. 2014; Meyer et al. 2018).

17.10 Perspectives

Changes in glycopattern that associate with age and age-related conditions have mainly been recorded in plasma and IgG *N*-glycome. Plasma/serum *N*-glycome, and more specifically, IgG *N*-glycome, seem to be good biomarkers for differentiation of healthy from unhealthy aging. This could hopefully be exploited in the future: for assessing the risk for ARDs and GSs, and detecting asymptomatic ARD and GS development, with the aim to intervene early, once reliable interventions are available. Moreover, the IgG glycome is proposed as a precise biomarker for the evaluation of lifestyle and medical interventions aimed at slowing down the rate of aging. A direct proof that IgG glycome alterations play a functional role in the aging process remains to be supplied, as well as the assessment of whether the rate of aging can be affected by targeted modulation of IgG glycome composition.

In the past 15 years, a lot of effort has been invested in the development and optimization of glycoanalytical methods, particularly in the area of high-throughput analysis, with impressive results. However, further optimization aiming to make the analysis simpler, more cost-effective, and accessible to a large number of people would be welcome. In particular, we would profit from a simplified sample collection, that is, usage of biological samples other than serum/plasma, such as dry blood spots (Ruhaak et al. 2012; Gudelj et al. 2015; Simunovic et al. 2019). Alternatively, we could turn to entirely non-invasive sampling, collecting specimens such as buccal swab (Speicher et al. 2020), saliva (Novak et al. 2005), and urine (Mistry and Kalia 2009), which have proven to be adequate sources of biological material for IgG glycoanalysis. Additionally, simplification of analytical procedures and method transfer to machines already approved for clinical testing, such as DNA sequencers (Laroy et al. 2006), is likely to make

plasma and IgG glycome analysis more approachable for everyday use in clinical labs.

In addition to their role as potential biomarkers, glycans and their precursors have been used as dietary supplements, with exciting effects on the rate of aging. The application of a dung beetle-derived GAG to old rats appeared to have an anti-aging effect measured by multiple parameters, such as oxidative damage, hepatocellular read-outs, protein carbonyl content, and malondialdehyde content (Ahn et al. 2017). In another study, the addition of GlcNAc into the growth medium of *Caenorhabditis elegans* slowed down aging in wild type animals and relieved symptoms of the neurotoxic disease (Denzel et al. 2014). Since GlcNAc is a precursor in the synthesis of *N*-glycans that are crucial for proper protein folding, the authors concluded that the induction of protein quality control mechanisms might prolong the lifespan and alleviate certain disease symptoms (Denzel et al. 2014). Still a long way from application in humans, these discoveries offer interesting ideas for possible uses of glycans aiming for healthy aging.

17.11 Conclusions

Glycosylation, just like aging, is regulated through a complex interplay of genetic, environmental, and other factors. Changes in glycosylation pattern, particularly of cell surface proteins, and serum/plasma and immunoglobulin G *N*-glycans, are associated with aging, and age-related and other diseases.

On a cellular level, aging is accompanied by β-galactosidase activity, which is extensively and routinely used for the detection of senescent cells in vitro. Additionally, cellular senescence (with erythrocytes as the most prominent example) is often characterized by a decreased content of terminal sialic acid on the cell surface, rendering these cells more susceptible to hepatic sequestration. By contrast, the surface of cancer cells is often hypersialylated, thus contributing to cancer genesis, growth, survival, and metastasis through various mechanisms.

Although serum/plasma and immunoglobulin G glycans undergo dynamic changes during aging, there is no direct proof that glycans play an effector role in the processes underlying aging. However, the inflammaging theory proposes that IgG glycosylation plays a functional role in aging through modulation of inflammation. IgG glycans are a good biomarker of healthy aging, which can be used to evaluate interventions aimed at slowing down the rate of aging. Several lifestyle and medical interventions have shown the capacity to change the IgG glycome composition, and the search for others is ongoing. Whether directed alteration of IgG glycopattern can affect the rate of aging remains to be seen.

While research on animal models could be particularly useful in this context, based on the first comprehensive study, mice do not appear to be a suitable model for exploring age-related changes in IgG glycosylation in humans. Due to large interstrain variability in IgG glycome composition, special attention should be paid to the selection of proper mouse strain for particular aging and intervention studies. More research is needed to confirm the initial results.

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Ethical Approval This work involves no human participants, animals, their data, or biological material, therefore no ethical approval was required.

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Correction to: Mucin-Type *O*-GalNAc Glycosylation in Health and Disease

Ieva Bagdonaite, Emil M. H. Pallesen, Mathias I. Nielsen, Eric P. Bennett, and Hans H. Wandall

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The chapter was inadvertently published with some colors illustrating important chemical differences lost on the right side of the Figure 2.1.

The correction has been incorporated by updating the revised figure 2.1 with the colors illustrating the chemical differences as seen in the next page.

The updated version of the chapter can be found at https://doi.org/10.1007/978-3-030-70115-4_2

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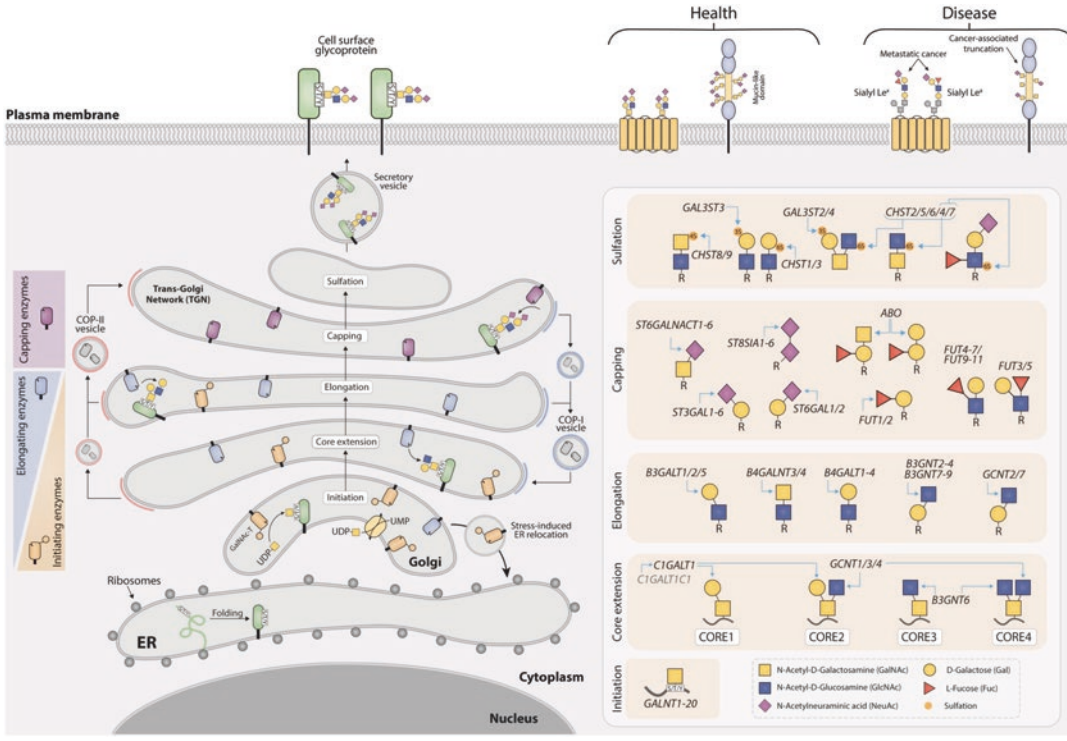

Fig. 2.1 *O*-GalNAc structures and biosynthesis
In healthy cells, the *O*-GalNAc biosynthesis pathway is restricted to the Golgi apparatus. A collection of different glycosyltransferases builds up the glycan structure as the carrier protein is transported through the different Golgi stacks and eventually ends up in secretory vesicles. The biosynthesis of the *O*-glycan structures can be divided into the following steps: 1) initiation, 2) core extension, 3) elongation, 4) capping, and 5) sulfation. The first initiation step is controlled by the members of the GalNAc-transferase enzyme family encoded by the *GALNT* genes. This family comprises 20 isoforms that catalyze the addition of a GalNAc monosaccharide to a threonine or serine (and in rare cases, tyrosine) residue in a newly synthesized protein. The subsequent addition of other sugars to the initial GalNAc will determine the type of core structure, with the four most common core structures depicted on the right. This panel also depicts the possible sugars that can be added to the extended *O*-glycan structure and the gene names for the respective enzymes. It is important to note that the enzymes are not limited to specific Golgi stacks. The gradient of initiating enzymes will decrease towards the Trans Golgi Network (TGN), while conversely, the gradient of branching and elongating enzymes will increase. The enzymes responsible for the final capping of the glycan structure are found in the outer stacks of the TGN. The resulting glycan structure can be affected by pathological conditions. For instance, malignant cancers are known for their lack of mature *O*-glycan structures on their surface proteins. Two other characteristic structures are the sialyl-Lewis-X and the sialyl-Lewis-A, which are upregulated in highly metastatic cancers

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