Mechanisms of disease: The human N-glycome ☆

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ABSTRACT

Background: The majority of human proteins are being modified by covalent attachment of complex oligosaccharides — glycans. Both glycans and polypeptide parts of a protein contribute to its structure and function, but contrary to polypeptide that is defined by the sequence of nucleotides in the corresponding gene, glycans are shaped by complex dynamic interactions between hundreds of enzymes, transcription factors, ion channels and other proteins.

Scope of review: An overview of current knowledge about the importance of N-glycans in normal human physiology and disease mechanisms, exemplified by IgG N-glycans.

Major conclusions: Recent technological development enabled systematic analysis of glycome composition in large epidemiological cohorts and clinical studies. However, the majority of these studies is still missing any glycomic component, and consequently also lacks this layer of biological information. Individual variation in glycosylation is potentially important for individualized disease risk, disease course and response to therapy. Evidence in support of this hypothesis is accumulating, but further studies are needed to enable understanding of the role of changes in protein glycosylation in disease.

General significance: Glycans are involved in virtually all physiological processes. Inter-individual variation in glycome composition is large, and these differences associate with disease risk, disease course and the response to therapy. This article is part of a Special Issue entitled "Glycans in personalised medicine" Guest Editor: Professor Gordan Lauc.

BOX: Small glossary of glycoscience

Glycan	Complex oligosaccharide composed of 10–15 monosaccharide residues. Can be covalently attached to proteins to make glycoproteins, or lipids to make glycolipids. Most glycans attached to proteins can be classified as N-glycans, attached through nitrogen of asparagine, or O-glycans, attached through oxygen of mainly serine or threonine.
Glycome	Set of all glycans in an organism/tissue/cell, or even of a single glycoprotein.
Glycoprotein	Molecular entity composed of a polypeptide and one or more glycans.
Glycoproteome	Set of all glycoforms of all glycoproteins in an organism/tissue/cell.
Glycosylation site	Stretch of several amino acids in a protein sequence recognized by the cell's glycosylation machinery as the place where certain glycans can be attached.
Alternative glycosylation	Addition of different glycans to the same glycosylation site on a polypeptide chain.
Glycoform	Protein isoform that differs only with respect to glycan(s) attached. An average glycoprotein comes in hundreds of different glycoforms.
Glycosyltransferase	Enzyme that adds a specific monosaccharide to a growing oligosaccharide.
Glycogene	Gene that codes for a glycosyltransferase or other proteins directly involved in biosynthesis and degradation of glycans.
Lectin	Protein with a functional carbohydrate recognition domain which binds specific glycan structures, regarding both monomer composition and spatial arrangement. (This does not include anti-carbohydrate antibodies.)

1. Glycans are essential for multicellular life

Glycans, alongside DNA, proteins and lipids, are one of four principal components of the cell. They are essential for multicellular life, as the complete absence of glycans is embryologically lethal [2]. They are the most abundant and diverse natural biopolymers, composed of saccharides that are typically added to nascent proteins and lipids within the cell secretory pathway (endoplasmatic reticulum and Golgi apparatus). The glycome represents the entire glycan repertoire of an organism/tissue/cell/protein, and is studied systematically by glycomics, a newly emerging field that has produced over 1.5 million scientific publications in the last 5 years [3]. Due to the large extent of variation in sugar monomer structure and inter-saccharide binding (bond type, branching) combined with the variation in glycan attachment sites the complexity of glycome exceeds that of the proteome by several orders of magnitude [4].

Glycans are assembled from monosaccharide residues through a carefully regulated enzyme-directed process of glycosylation. In contrast to polypeptides, which are defined by the sequence of nucleotides in the corresponding genes, glycans are shaped by complex dynamic interactions between hundreds of enzymes, transcription factors, ion channels and other proteins [5,6], Since genetic background (reflected in the proteins involved in glycan synthesis) and environmental factors integrate at the level of glycan biosynthesis [7], the glycome represents a form of cellular memory, which modulates current cellular physiology on the basis of past events in the cell [8,9].

The glycosylation machinery is influenced by both genetic and environmental factors. Severe glycan deficiencies caused by mutations in the early stages of the glycan biosynthesis pathway (responsible for core glycan structures) cause deficiencies in cellular growth and function and lead to a variety of debilitating diseases called congenital disorders of glycosylation [10]. In contrast, great diversity in the terminal glycan antennae is common. A well-recognized example of this is the existence of AB0 blood groups, which differ between individuals in oligosaccharide antigens attached to the proteins and lipids. The difference between these sugar moieties stems from three allelic variants of a single glycosyltransferase gene, each allowing for the synthesis of a different carbohydrate antigen.

Glycans participate in numerous molecular processes, including protein folding, cell-adhesion, molecular trafficking, signal transduction, modulation of receptor activity and others [6]. As such, they play a major role in all fundamental functions of the multicellular organism, including the immune system, particularly regarding mucosal barrier maintenance, "self" vs. "non-self" discrimination and behavior of immune cells. Human cells are covered with a dense layer of glycans attached to membrane proteins and lipids — the glycocalyx (literally meaning "sugar coat"), a structure at least 10, and sometimes even 1000 times thicker than the cellular membrane itself (Fig. 1). Glycocalyx represents a cell's fingerprint, a type of identifier that the human body uses to distinguish between "self" and "non-self". Foreign glycan patterns present on transplanted tissues, invading organisms, but also own diseased cells are recognized by soluble and cell membrane glycan receptors that activate innate immune response mechanisms.

Given the fact that glycans participate in many biological processes, it is not surprising that molecular defects in glycan synthesis are recognized as direct causes of an increasing number of diseases [10]. Many specific glycan variants are now considered disease markers and represent diagnostic as well as therapeutic targets [11–13].

Glycans can be covalently attached to proteins to make glycoproteins, or lipids to make glycolipids. Two major groups of branched glycans attached to various amino acids in the glycoprotein backbone comprise: N-linked glycans — attached to the nitrogen of asparagine or arginine side chains; and O-linked glycans — attached to the oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline side chains. Another group of glycoproteins are proteoglycans, that consist of long, unbranched often sulfated carbohydrate structures (glycosaminoglycans), attached to serine or threonine of protein backbone. Due to the vast expanse of the topic and the fact that they are present in about 90% of all glycoproteins [4], this review focuses on N-glycans as the major and best studied class of carbohydrate protein modifications. However, it should be noted that other types of glycosylation, in particular O-glycosylation, proteoglycans and glycolipids are also very important and should be included in large population studies that look at disease mechanisms.

- *"Glycans are directly involved in the pathophysiology of every major disease."*
- *"Additional knowledge from glycoscience will be needed to realize the goals of personalized medicine and to take advantage of the substantial investments in human genome and proteome research and its impact on human health."*
- *"Glycans are increasingly important in pharmaceutical development."*

US National Academies, 2012 [1]

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2. Variation in protein glycosylation has functional significance

Nearly all human membrane and secreted, as well as numerous intracellular proteins are co- and post-translationally modified by the covalent addition of complex oligosaccharides [5]. Glycoproteins therefore represent more than a half of all proteins, such as most serum proteins [4], with their glycan parts often playing an essential functional role. A comprehensive report endorsed by the US National Academy of Sciences stated that glycans serve as "on and off" switches that modulate the functions of glycoproteins, the proteome *predicting* the phenotype, but glycoproteome actually *being* the phenotype [1].

In contrast to the linear nature of nucleic acids (DNA, RNA) and proteins, glycans attached to proteins are non-linear branched molecules. Due to their structural complexity and methodological difficulties associated with the analysis of small amounts of glycans in diagnostic samples, knowledge about the role of glycans in disease mechanisms lags significantly behind the knowledge about the role of genes and proteins. However, as more information about protein glycosylation emerges, it is becoming increasingly clear that glycosylation is strictly regulated and that glycan attachment to proteins is of paramount physiological significance [5]. Glycan parts of glycoproteins are involved in virtually all biological processes, from fertilization and embryogenesis, through cell proliferation, differentiation and development, to immunity and aging.

Despite their different biosynthetic origin, both polypeptide and glycan parts of glycoproteins participate as constitutive molecular entities in the function of a glycoprotein (Fig. 1). Therefore, without the knowledge about the structure and function of glycan parts of glycoproteins, many aspects of their biology and involvement in disease mechanisms will remain elusive. In principle, glycans contribute to protein function in three ways: (1) representing an integral part of the protein and thus being essential for protein's proper structure and/or function — this refers to protein folding, solubility and stability; (2) fine-tuning protein structure and thus modulating its function through alternative glycosylation (addition of different glycans to the same attachment site on the polypeptide chain) in order to tailor it to specific physiological requirements; and (3) forming independent binding sites for glycan-specific receptors called lectins. Through all three modes of action glycans are directly involved in various disease mechanisms.

This review presents examples of the paramount importance of glycans in immunity, cancer and activity of biological therapeutics.

3. Glycans are necessary for interaction of pathogenic and commensal microbes with host cells

Pathogen infection typically begins with host glycan recognition and binding by one or more of viral, bacterial, or protozoan numerous lectins. The first identified microbial lectin was the hemagglutinin from the influenza virus. A glycoprotein itself, its name originates from the ability to crosslink red blood cells by binding to terminal sialic acids on their surface. Influenza hemagglutinin is one of the best studied lectins and its specific recognition of distinct glycan structures, namely the type of covalent bond between 2 monosaccharide units (sialic acid-2,3-galactose, vs. sialic acid-2,6-galactose) on the surface of host target cells is the main barrier requiring genetic conversion for influenza viruses to cross between species [14]. The importance of this feature became apparent during the spreading of the most pathogenic bird influenza virus H5N1 in the last 15 years, raising global public concern over a possible human pandemic threat. Interestingly, both letters in the name of the virus are related to glycans, thus underlining the importance of glycans in viral infection. The H stands for the aforementioned hemagglutinin, necessary in the initial stages of viral infection, while the N stands for neuraminidase, i.e. sialidase, necessary for the release of viral progeny from infected cells by cleaving terminal sialic acid residues on host glycoproteins.

The specificity of rotaviruses, an important cause of childhood death (from dehydration secondary to acute gastroenteritis) in developing countries, also lies in the specificity of their glycan attachment. Host susceptibility to specific human rotavirus strains and disease pathogenesis appear to be influenced by the expression of different ABO blood group antigens [15], also recognized as susceptibility and cell attachment factors for other gastric pathogens like *Helicobacter pylori* [16] and noroviruses [17]. It is generally thought that the development of ABO polymorphic surface presentation system has an evolutionary advantage in limiting the spread of pathogens that specifically recognize its glycoantigens. Even when pathogens do not bind directly to ABO glycans, their alteration of the presentation of cognate ligands on the cell surface can modulate binding of pathogens such as *Plasmodium falciparum* malarial parasite and limit their transmission between individuals [18].

The inhibition of pathogen binding to host cell ligands is an important element of protection against microbial pathogens. In breastfed newborns it is interestingly rendered by a complex repertoire of glycans that represents the third largest component of human mother's milk. The glycome composition in mother's milk is therefore found to be significantly associated with the risk of infectious diseases in breastfed infants [19].

Long after the discovery of human glycans glycoscience was unaware of the existence of glycosylation machinery in bacteria. It is now known that N- and O- glycans on the surface of many pathogenic bacterial genera (*Campylobacter*, *Helicobacter*, *Clostridium*, *Haemophilus*, *Escherichia*, *Neisseria*, *Mycobacterium*, *Streptomyces* etc.) are indispensable for their motility and adherence to surfaces, therefore playing a major role in their virulence and representing putative therapeutic targets [20]. In symbiotic bacteria glycans are extensively involved in homeostasis maintenance, playing a key role in symbionts' colonizing capacity, survival advantage and attenuation of the host's immune response [21]. In case of intestinal microbiota they at the same time serve as a nutrient foundation that helps organize initial colonization of different regions of the postnatal intestine. [22] Modulation of the intestinal epithelial glycome is a fascinating example of the role that host glycans play in the interaction between higher organisms and microbes. The exact mechanisms of this phenomenon are still being elucidated, but it has been shown that signals from specific non-pathogenic commensal bacteria affect the host's glycosylation machinery and instruct it to produce glycans that promote successful symbiosis [22,23].

4. Inflammation is initiated by glycans

Selectins are a family of cell surface lectins originally identified as key initiators of inflammation [24–26]. All selectins function by binding to specific glycoprotein and glycolipid ligands on the cell surface in a calcium-dependent manner [27]. They play a significant and a well-documented role in leukocyte recruitment and migration to sites of inflammation, initiating tethering and rolling of circulating leukocytes that leads to their activation, adhesion and subsequent extravasation into tissues, as well as signal transduction. This makes selectins essential for mounting a functional immune response, which includes immune surveillance and inflammation. Their importance has been further recognized in various processes requiring cell adhesion, including blood cell homeostasis, metastasis [28] and maternal-fetal interactions [29].

Three members of the selectin family have been identified, L-, P-, and E-selectins. L-selectin (CD62L, LAM-1, LECAM-1) is constitutively expressed on all classes of peripheral blood leukocytes and is rapidly shed by proteolytic cleavage upon their activation [30]. It functions as a homing receptor, mediating binding of lymphocytes to high endothelial venules of peripheral lymph nodes, which makes it indispensable for constitutive lymphocyte trafficking. It is also included in leukocyte homing to the inflammation sites after activation. P-selectin (CD62P, LECAM-3, GMP-140, PADGEM) is expressed and translocated to the cell surface within minutes of an inflammatory stimulus in activated platelets and inflamed endothelial cells. E-selectin (CD62E, ELAM-1, LECAM-2) is expressed on endothelial cells after *de novo* synthesis, within a few hours of activation. P-selectin (in acute injury) and E-selectin (in inflammatory conditions) mediate the initial leukocyte capture and rolling along the wall of post-capillary and collecting venules [31].

Dysregulation of selectins or their glycoprotein ligands can lead to exacerbation of a variety of disease processes, including atherosclerosis, restenosis (recurrence of stenosis), deep venous thrombosis and tumor metastasis, while pharmacological blockade of selectins has been demonstrated to ameliorate disease pathology [32]. Since selectins function through recognition of glycans on the cell surface, any alterations in the presentation of glycans can have significant effects on this interaction. For example, the presence of monosaccharide fucose in the form of Sialyl Lewis X (sLeX or CD15s) or sialyl Lewis A (sLeA) is required for proper binding and subsequent selectin-dependent leukocyte adhesion and trafficking [33]. 90% of cellular fucose, necessary for selectin ligand synthesis, is supplied by a fucose-generating FX enzyme. Its importance in lymphocyte migration is manifested in knock-out mice, which display an immunodeficient phenotype due to impaired leukocyte recruitment, similar to patients with the rare congenital disease leukocyte adhesion deficiency type II (LAD II) [34]. These patients suffer from severe recurrent bacterial infections caused by defective fucose processing that prevents the production of functional fucosylated selectin ligands on the cell surface [35,36].

The effects of the wide inter-individual variation in protein glycosylation patterns in human populations [37,38] on the function of selectins and their participation in disease mechanisms remain to be elucidated.

5. Glycosylation regulates antibody function

Almost all key molecules involved in the processes of innate and adaptive immunity, including immunoglobulins (Ig) of all five classes, are glycoproteins [39]. Depending on the class, carbohydrate content makes up to 15% of antibody weight, and is critical for the appropriate functioning of all immunoglobulins. Due to its extensive involvement in immunological processes, IgG is one of the mostly studied glycoproteins and represents an excellent example of protein function modulation by alternative glycosylation. While the Fab parts of IgG are responsible for the recognition of antigenic structures, the Fc part executes further actions for the removal or destruction of recognized objects through interaction with different Fcγ and other
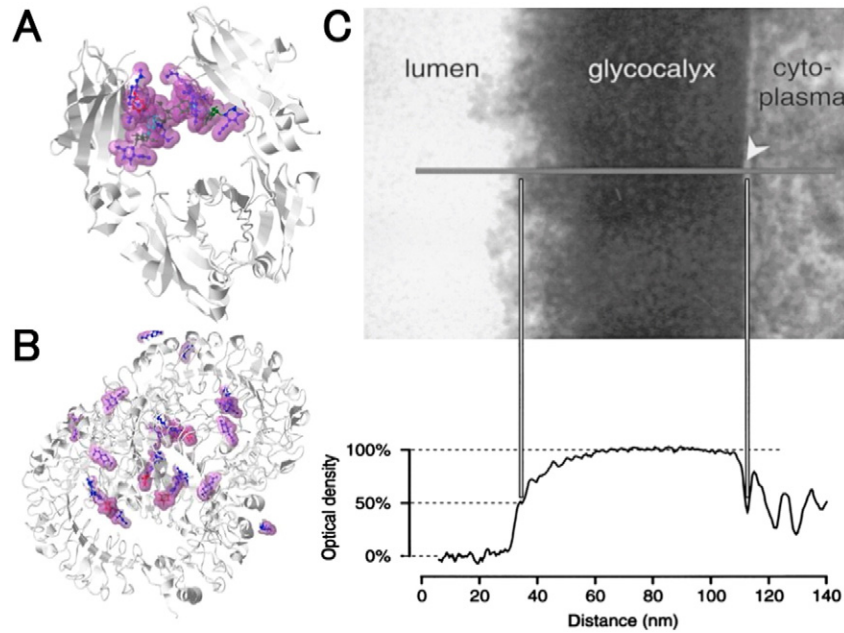
Fig. 1. Glycans are important structural and functional component of the majority of proteins. Two examples of glycoprotein structure: Fc fragment of immunoglobulin G (A) and toll like receptor 8 (B) are provided in the form of molecular models. The polypeptide backbone is shown in gray, and glycans in color. Despite their different biosynthetic origin, both glycan and polypeptide parts participate in their structure and function. Glycans are particularly important at the cell membrane where they form a glycocalyx (C), a complex layer of glycoproteins and proteoglycans that is 20–30 times thicker than the phospholipid bilayer of the cell membrane (from Vogel at al, *Journal of Cerebral Blood Flow* & *Metabolism* 20:1571, 2000).
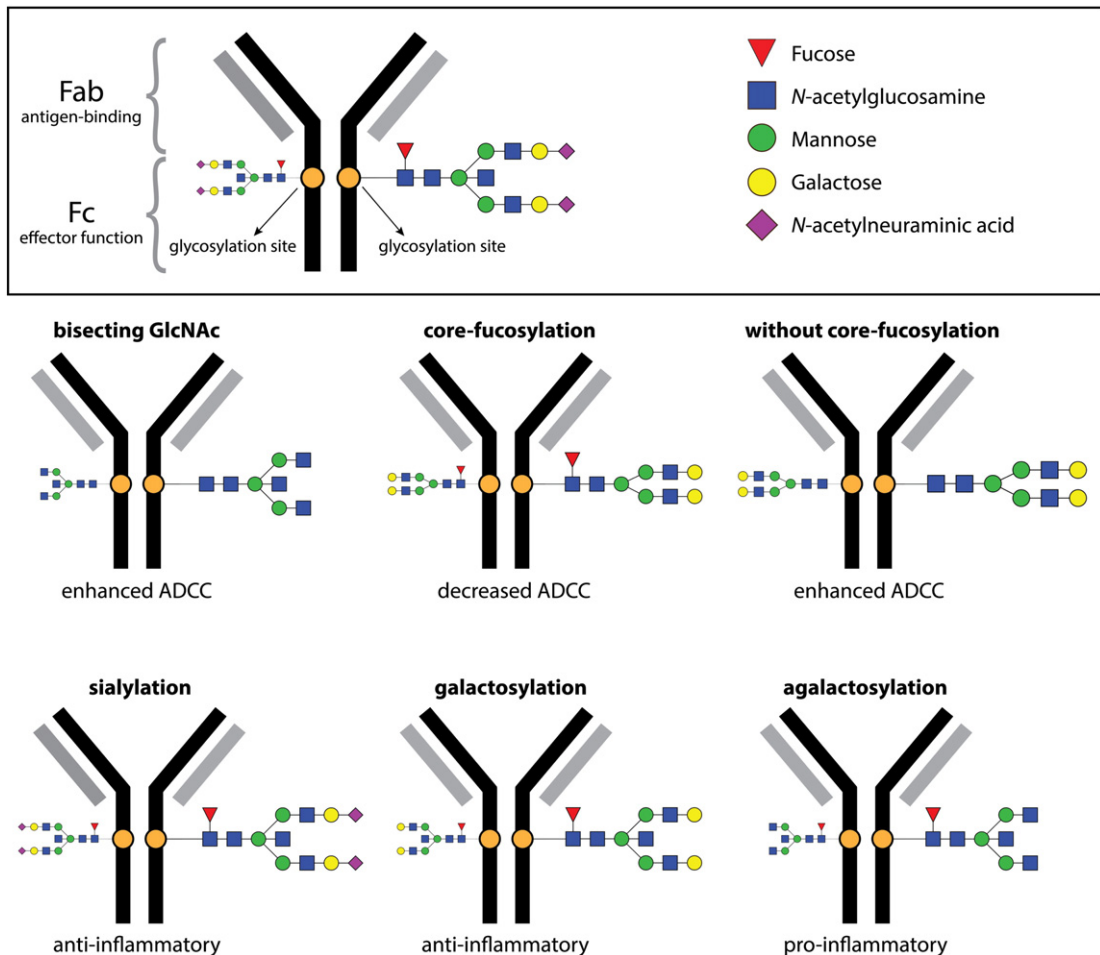
Fig. 2. Functional implications of alternative glycosylation of IgG.

receptors. Each IgG heavy chain carries a single covalently attached bi-antennary N-glycan that is an essential structural component of the Fc region. The attachment of different glycans can significantly change conformation of the Fc with dramatic consequences for IgG effector functions [40] (Fig. 2).

Population studies have found high levels of inter-individual variation in total plasma and immunoglobulin G (IgG) glycome [37,41]. Both total plasma and IgG glycome composition have been shown to be longitudinally quite stable in homeostatic conditions [42]. However, they change significantly but at a slow pace with age [37,43], and can be rapidly altered in situations of disturbed homeostasis [44]. The variety and dynamic in glycome composition can thus represent both an evolutionary advantage and a means to quickly respond to environmental stimuli.

Over 95% of circulating human IgG contains a fucose residue attached to the first *N*-acetylglucosamine in the IgG glycan core (core-fucose) [41]. This is an unusual feature of IgG, since the majority of other plasma proteins are not core-fucosylated [37]. The presence of core-fucose is known to dramatically reduce IgG binding to FcγRIIIA [45–47], an activating Fc receptor specific for IgG Fc region and expressed on the surface of innate immune cells, such as natural killer (NK) cells and macrophages. It initiates antibody dependent cellular cytotoxicity (ADCC) by NK cells and antibody dependent cellular phagocytosis (ADCP) by macrophages upon antigen binding. The presence of a high proportion of IgG which is core-fucosylated therefore represents a "safety switch" which attenuates potentially harmful ADCC activity [48]. By contrast, ADCC induced by non-fucosylated IgG seems to be one of the primary modes of function of therapeutic anti-cancer monoclonal antibodies, since IgG molecules lacking core-fucose are over 100 times more effective in initiating ADCC [45,49,50]. Interestingly, high affinity binding between FcγRIIIA and antibodies lacking core fucose requires unique carbohydrate-carbohydrate interactions between IgG glycans and glycans attached to FcγRIIIA [51,52], further reinforcing the importance of glycosylation in the regulation of ADCC.

Another important structural feature of IgG glycans is the addition of galactose, which seems to be regulated in a very complex manner. It changes gradually in association with physiological factors (such as age and hormonal status), but can also change rapidly in an "on and off mode" in acute inflammation [44]. A decrease in IgG galactosylation is also noted in many autoimmune and inflammatory diseases, where it probably plays a functional role causing a decrease in the immunosuppressive potential of IgG. Fc galactosylation has recently been found a prerequisite for the efficient association between FcγRIIB and dectin-1, leading to IgG anti-inflammatory activity [53]. Likewise immune complexes rich in galactose residues have been found to inhibit the pro-inflammatory activity of the complement component C5a [54, 55]. This may represent another mechanism by which decreased IgG galactosylation participates in the pathology of autoimmune and inflammatory diseases.

The presence of glycans with terminal sialic acid converts IgG function from pro-inflammatory to anti-inflammatory in some mouse models. This is confirmed by the finding that the hyper-sialylated Fc fraction, recognized by the DC-SIGN (CD209) receptor, is responsible for the anti-inflammatory property of intravenous immunoglobulins administered at high doses (g/kg) [56,57]. The anti-inflammatory and immunosuppressive activities of highly sialylated IgG molecules are thought to be one of the key elements in immune homeostasis and prevention of autoimmune and inflammatory diseases [55,58].

6. Protein glycosylation in autoimmune and inflammatory diseases

Autoimmune diseases are triggered by aggressive responses of the adaptive immune system to self-antigens, resulting in tissue damage and pathological sequelae [59]. Among other mechanisms, autoantibodies are responsible for the chronic inflammation and destruction of healthy tissues by interaction with the complement network and Fc

receptors on innate immune effector cells. [60] The class/subclass and glycosylation status, particularly in the case of IgG, are important in determining the pathogenicity of autoantibodies in autoimmune diseases [61]. Removal of IgG glycans leads to the loss of pro-inflammatory activity, suggesting that in vivo modulation of antibody glycosylation might be a strategy to interfere with autoimmune processes [60].

Decreased galactosylation of IgG in rheumatoid arthritis (RA) is well established [62,63]. It has been shown that incompletely galactosylated IgG can activate complement via the mannose-binding protein, thus taking part in the underlying pathological mechanism of rheumatoid arthritis via lectin complement pathway [54]. Decreased IgG galactosylation has also been reported to precede the development of RA [64,65], indicating that this more pro-inflammatory form of IgG may be either a predisposing factor, or a functionally relevant change that contributes to the RA development. Decreased IgG galactosylation has also been reported in inflammatory bowel disease (IBD) [66,67] and systemic lupus erythematosus (SLE) [68,69]. In SLE, changes in IgG glycosylation also associated with symptom severity. The cross-sectional nature of these studies did not allow for any inference of causality, but the significant pleiotropic effects of a number of genes on both IgG glycome and IBD and/or SLE, as well as the very high heritability of the IgG glycome [70] suggest that glycoforms which decrease immunosuppressive activity of IgG may be a predisposing factor for autoimmune and inflammatory disease.

The leukocyte and endothelial glycocalyx is of paramount importance in the process of inflammation underlying the autoimmune diseases' pathogenesis. Membrane glycoproteins show very complex and sophisticated glycosylation patterns and their glycans play numerous important roles in the immune response [71], such as leukocyte activation, migration and tissue infiltration. Altered protein glycosylation and antibodies that recognize endogenous glycans have been associated with various autoimmune diseases. For example, mouse strains with deficiencies in glycan branching, are hypersensitive to autoimmune disease [72], while modification of sialic acids on membrane glycoproteins by sialic acid acetylesterase is important in peripheral B cell tolerance [73] and functionally deficient germline variants of this gene represent a strong genetic link to susceptibility in some relatively common human autoimmune disorders [74].

7. Glycosylation in cancer

Since hundreds of genes are involved in glycan biosynthesis [75], this process is inherently sensitive to alterations in cellular physiology. Consequently, extensive genetic alterations that are associated with malignant transformation are inevitably accompanied by changes in protein glycosylation, as evidenced by changed glycoprofiles of many cancer markers. Glycans are known to play a role in tumor proliferation, invasion, haematogenous metastasis and angiogenesis [11]. Some aspects of protein glycosylation (glycan branching in particular) appear to be of great importance for cancer progression and metastasis, cell to cell contact, and epithelial-mesenchymal transition [12]. The epigenetic regulation of glycosyltransferases in cancer cells results in the creation of novel glycan structures that appear to be one of the mechanisms used by cancer cells to evade the host immune response [76]. A recent comprehensive study of genetic variants that mediate breast cancer metastasis to the brain identified α-2,6-sialyltransferase ST6GALNAC5 as the key gene specifically mediating brain metastasis [77]. Normally restricted to the brain, the expression of ST6GALNAC5 in breast cancer cells enhances their adhesion to brain endothelial cells and their passage through the blood–brain barrier. This highlights the potential wider role of cell-surface glycosylation in organ-specific metastatic interactions. The majority of currently used protein cancer biomarkers are actually glycoproteins and their glycosylation is significantly altered in cancer (Table 1). However, despite significant biomarker potential of changes in glycosylation [78,79], this type of analytics still needs to find its way to routine clinical practice. Acknowledging the importance of

Table 1

Glycosylation of clinically used cancer biomarkers. The majority of FDA-approved protein tumor markers currently used in clinical practice are actually glycoproteins and for many of them it was shown that their glycosylation changes in cancer. However, despite significant biomarker potential, [78,79] changes in cancer biomarker glycosylation are currently not used in routine clinical practice.

Biomarker	Cancer type	Reference showing relevance of glycosylation
Prostate specific antigen (PSA)	Prostate	Gilgunn, S., Conroy, P. J., Saldova, R., Rudd, P. M., and O'Kennedy, R. J. (2013) Aberrant PSA glycosylation—a sweet predictor of prostate cancer. *Nature reviews. Urology* 10, 99–107.
Alpha-fetoprotein (AFP)	Liver cancer and germ cell tumors	Sato, Y., Nakata, K., Kato, Y., Shima, M., Ishii, N., Koji, T., Taketa, K., Endo, Y., and Nagataki, S. (1993) Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N. Engl. J. Med. 328, 1802–1806.
Beta-human chorionic gonadotropin (Beta-hCG)	Choriocarcinoma and testicular cancer	Lempiainen, A., Hotakainen, K., Blomqvist, C., Alfthan, H., and Stenman, U. H. (2012) Hyperglycosylated human chorionic gonadotropin in serum of testicular cancer patients. Clin Chem 58, 1123–1129.
MUC-1 (CA15-3/CA27.29)	Breast cancer	Brockhausen, I., Yang, J. M., Burchell, J., Whitehouse, C., and Taylor-Papadimitriou, J. (1995) Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. Eur J Biochem 233, 607–617.
Carbohydrate antigen 19.9 (CA19-9)	Pancreatic cancer, gallbladder cancer, bile duct cancer, and gastric cancer	Yue, T., Goldstein, I. J., Hollingsworth, M. A., Kaul, K., Brand, R. E., and Haab, B. B. (2009) The prevalence and nature of glycan alterations on specific proteins in pancreatic cancer patients revealed using antibody-lectin sandwich arrays. Mol Cell Proteomics 8, 1697–1707.
MUC16 (CA-125)	Ovarian cancer	Jankovic, M. M., and Milutinovic, B. S. (2008) Glycoforms of CA125 antigen as a possible cancer marker. Cancer biomarkers : section A of disease markers 4, 35–42.
Carcinoembryonic antigen (CEA)	Colorectal cancer and breast cancer	Saeland, E., Belo, A. I., Mongera, S., van Die, I., Meijer, G. A., and van Kooyk, Y. (2012) Differential glycosylation of MUC1 and CEACAM5 between normal mucosa and tumor tissue of colon cancer patients. Int. J. Cancer 131, 117–128.
Chromogranin A (CgA)	Neuroendocrine tumors	Gadroy, P., Stridsberg, M., Capon, C., Michalski, J. C., Strub, J. M., Van Dorsselaer, A., Aunis, D., and Metz-Boutigue, M. H. (1998) Phosphorylation and O-glycosylation sites of human chromogranin A (CGA79-439) from urine of patients with carcinoid tumors. J. Biol. Chem. 273, 34,087–34,097.
HE4	Ovarian cancer	Hua, L., Liu, Y., Zhen, S., Wan, D., Cao, J., and Gao, X. (2014) Expression and biochemical characterization of recombinant human epididymis protein 4. Protein expression and purification 102, 52–62.
Thyroglobulin	Thyroid cancer	Di Jeso, B., Formisano, S., and Consiglio, E. (1999) Depletion of divalent cations within the secretory pathway inhibits the terminal glycosylation of complex carbohydrates of thyroglobulin. Biochimie 81, 497–504.
Plasminogen activator inhibitor (PAI-1)	Breast cancer	Gils, A., Pedersen, K. E., Skottrup, P., Christensen, A., Naessens, D., Deinum, J., Enghild, J. J., Declerck, P. J., and Andreasen, P. A. (2003) Biochemical importance of glycosylation of plasminogen activator inhibitor-1. Thromb Haemost 90, 206–217.

glycans in malignant transformation, The National Cancer Institute has begun an initiative to discover, develop and clinically validate glycan biomarkers for cancer (http://glycomics.cancer.gov/) [80–85].

8. The importance of glycans for therapeutics

Many recombinant pharmaceuticals, including therapeutic monoclonal antibodies, are glycoproteins (Table 2), and their specific glycoforms are the key to their bio-activity and half lives in circulation. Improper glycosylation, like, for example, the presence of *N*-glycolylneuraminic acid in recombinant therapeutic glycoproteins can

Table 2

Top 10 best selling drugs in 2008 and 2013. Eight out of 10 best selling drugs in Europe are glycoproteins (IMS Health,MIDAS, MAT June 2013)

	2008	2013
1	Lipitor (high cholesterol)	Humira (arthritis)
2	Seretide (asthma)	Seretide (asthma)
3	Plavix (blood clotting)	Enbrel (autoimmune diseases)
4	Herceptin (breast cancer)	Herceptin (breast cancer)
5	Enbrel (autoimmune diseases)	Mabthera (leukaemia)
6	Zyprexa (schizophrenia and bipolar disorder)	Remicade (arthritis)
7	Lovenox (deep vein thrombosis)	Lovenox (deep vein thrombosis)
8	Glivec (cancer)	Avastin (cancer)
9	Pantozol (stomach acid)	Lucentis (age-related macular degeneration)
10	Symbicort (asthma and COPD)	Lyrica (epilepsy)

cause severe adverse reactions in some individuals [86]. Glycans attached to Fc domain modulates effector functions of monoclonal antibodies and tuning of glycosylation to desired effector functions can improve efficacy of the drug. For example, it was recently shown that glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab [87]. Recombinant erythropoietin is another example where glycosylation is of paramount importance for therapeutic activity. Glycans constitute about 40% of its molecular weight and differentiate between different glycoprotein variants sharing the same polypeptide sequence. It was glycan engineering (mostly increasing sialic acid content) that led to the production of the improved hyperglycosylated variant: novel erythropoiesis stimulating protein with prolonged serum half-life, resulting in increased bio-activity and reduced administration frequency [88]. For example, the introduction of two additional N-glycosylation sites increased the half-life of darbepoetin from 7 to 8 h to approximately 22 h, thus making this glyco-engineered form of erythropoietin a more potent and much more convenient drug [89]. It is to be expected that the potential of glyco-engineering strategies will be further used in the future for the optimized production of glycoconjugates for therapeutic and vaccination purposes.

The anti-flu virus blockbuster drugs, Relenza™ and Tamiflu™ are analogs of sialic acids that inhibit the influenza virus neuraminidase and hence the transmission of the virus [90]. Natural heparin, a sulfated glycosaminoglycan, and chemically defined synthetic heparin oligosaccharides, are widely used in the treatment and prophylaxis of multiple thrombosis-related diseases. Hyaluronic acid, a non-sulfated glycosaminoglycan, is used in the treatment of arthritis. Recently, the first sialylated intravenous immunoglobulin preparation with consistent anti-inflammatory potency has been found suitable for clinical development [91].

The other aspect of importance of glycans for therapeutics is their role in the individualization of patient's response to drug. Glycosylation regulates the membrane half-life of numerous membrane receptors [92], including GLUTs [93], cytokine receptors [94], TGF-beta receptor [95], EGF receptors [96], GABAA receptors [97] and others. Many drugs either bind directly on these receptors, or include them in the signaling

pathway. The existence of multiple common polymorphisms in the glycosylation pathway results in specific glycoforms modulating receptor function and consequently drug efficacy in a given individual. For example, it has been recently shown that success of IVIG therapy in Kawasaki disease depends on the glycosylation of host IgG [98]. Another example of importance of individual differences in glycosylation is the finding that spontaneous control of HIV and improved antiviral activity are associated with a dramatic shift in the global antibody-glycosylation profile toward agalactosylated glycoforms [99]. New methods for high throughput screening of the individual's glycome appear to be a promising tool for patient stratification, which could contribute to the field of precision medicine through enabling individualization of the therapeutic approach according to an individual's glycomic profile [100]. Extended research is still required on glycan analysis and modulation methods aiming at production of more effective and reliable therapeutics.

9. Conclusions

Glycans are one of the four principal components of the cell and as such play important roles in most physiological processes and diseases. Knowledge about glycans and their functions in health and disease lags significantly behind knowledge about nucleic acids and proteins. However, the field of glycoscience is developing rapidly and awareness about the importance of glycans in disease mechanisms is growing. Since their structures are not hard-wired in the DNA sequence, the principal role of glycans seems to be the modulation of biological interactions - for example, through changing the function of immunoglobulins or the cell surface half-life of specific membrane receptors. Some of the most important drugs on the market are glycoproteins and glycan analysis and modulation is now considered a necessary step in the production of bio-therapeutics. Population studies have demonstrated that inter-individual differences in glycosylation are large and these differences may explain at least some elements of individual variation in disease course or response to therapy. Any study attempting to understand a disease mechanism, which does not account for individual variations in protein glycosylation, will therefore miss an essential part of its underlying biology. With its potential to classify patients according to disease predisposition, prognosis, and response to therapy, glycan analysis today has an immense capacity to contribute to the evolving field of precision medicine.

Conflict of interests statement

Gordan Lauc is the founder and owner of Genos Ltd — a private research organization that specializes in high-throughput glycomic analysis. Marija Pezer is employee of Genos Ltd. Other authors declare no conflict of interest.

Acknowledgements

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