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REVIEW ARTICLE

Altered N-glycosylation profiles as potential biomarkers and drug targets in diabetes

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N-glycosylation is a ubiquitous protein modification, and N-glycosylation profiles are emerging as both biomarkers and functional effectors in various types of diabetes. Genome-wide association studies identified glycosyltransferase genes as candidate causal genes for type 1 and type 2 diabetes. Studies focused on N-glycosylation changes in type 2 diabetes demonstrated that patients can be distinguished from healthy controls based on N-glycome composition. In addition, individuals at an increased risk of future disease development could be identified based on N-glycome profiles. Moreover, accumulating evidence indicates that N-glycans have a major role in preventing the impairment of glucose-stimulated insulin secretion by maintaining the glucose transporter in proper orientation, indicating that interindividual variation in protein N-glycosylation might be a novel risk factor contributing to diabetes development. Defective N-glycosylation of T cells has been implicated in type 1 diabetes pathogenesis. Furthermore, studies of N-glycan alterations have successfully been used to identify individuals with rare types of diabetes (such as the HNF1A-MODY), and also to evaluate functional significance of novel diabetes-associated mutations. In conclusion, both N-glycans and glycosyltransferases emerge as potential therapeutic targets in diabetes.

Keywords: glycosyltransferase; HNF1A-MODY; N-glycosylation; type 1 diabetes; type 2 diabetes

Main types of diabetes and current diagnostic criteria

Diabetes is a chronic disease characterized by hyperglycaemia, with accompanying risk of long-term complications which affect nervous system, kidney, eyes and heart [1,2]. It is estimated that over 420 million people have diabetes, with prevalence in adult population approaching 10% in the developed world [3]. Diabetes caused 10.7% of all global deaths among people aged between 20 and 79 years in 2017 [3]. Half of all adult people with diabetes are estimated to remain undiagnosed [4]. In the United States, 41.7% of individuals with previously undiagnosed diabetes developed chronic kidney disease [5]. In the cohort of individuals with coronary artery disease, measures including total cholesterol, triglycerides, BMI and fasting blood glucose were all higher in individuals with undiagnosed diabetes than in those with established diabetes [6]. One study evaluated total economic costs of undiagnosed diabetes in the United

Abbreviations
ACR, albumin-to-creatinine ratio; ADCC, antibody-dependent cellular cytotoxicity; AGP, alpha-1-acid glycoprotein; CRP, C-reactive protein; CTLA-4, cytotoxic T-lymphocyte protein 4; eGFR, estimated glomerular filtration rate; FUTs, fucosyltranferases; GMDS, GDP-mannose-4,6-dehydratase; GWAS, genome-wide association studies; HBP, hexosamine biosynthesis pathway; hs-CRP, high-sensitivity C-reactive protein; IgG, immunoglobulin G; LacNAc, *N*-acetyllactosamine; MODY, maturity-onset diabetes of the young; TCRs, T-cell receptors; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

States for the year 2012 on more than 32 billion USD [7].

Widely accepted classification categorizes main types of diabetes into type 2 diabetes, type 1 diabetes and gestational diabetes [3,8]. The most common type of diabetes is type 2 diabetes, which accounts for approximately 90% of diabetes cases [3,9–11]. In high-income countries, type 1 diabetes accounts for 7–12%, and other types of diabetes for 1–3% of diabetes cases [3,9–12]. Incidence of type 1 diabetes varies hugely depending on regions [13–15]. Global prevalence of hyperglycaemia in pregnancy in 2017 was estimated to be 16.2%, of which 86.4% cases were due to gestational diabetes [3]. Less common types of diabetes include monogenic diabetes, such as neonatal diabetes and maturity-onset diabetes of the young (MODY), and secondary diabetes [3].

Diabetes diagnosis is based on plasma glucose concentration, which is measured either in a sample taken after fasting or 2 h after 75-g glucose intake, or on HbA1c measurements [3,16,17]. HbA1c, glycated haemoglobin which is formed by the nonenzymatic glycation of the haemoglobin A, provides information on the average blood glucose concentration during the last 2–3 months, and as such represents a gold standard for evaluating glycaemic control in people with diabetes [18]. Yet, it can often be difficult to assign a type of diabetes to an individual, especially as classification is subject to circumstances at the time of diagnosis [19,20]. For example, in some individuals with the classical appearance and clinical diagnosis of type 2 diabetes, there is evidence of islet autoimmunity [21], and they may have a slowly progressive type of autoimmune diabetes.

N-glycosylation and its implication in diabetes

Over 40 years ago it was shown that levels of specific glycans bound to serum proteins were increased in patients with diabetes, of which only a small proportion could be explained by the increase in the levels of glycoproteins carrying these glycans [22], suggesting a direct role of glycans in the pathophysiology of diabetes, which was subsequently confirmed for some specific proteins [23]. Here, it is important to stress that glycosylation should not be mistaken for glycation, since glycosylation is a complex enzymatic process strictly regulated by a network of glycosyltransferases, glycosidases, transcriptional factors, sugar nucleotides and other molecules [24]. Glycation, on the other hand, represents the nonenzymatic reaction of reducing sugar and protein [25], such as the one described for glycated haemoglobin [18].

Proper glycosylation is important for correct protein folding, cell structure maintenance, receptor–ligand interactions, cell signalling, cell–cell recognition and immune defence [26]. Development of high-throughput glycan analyses was challenging, especially due to complex and highly branched structure representative of glycans [26]. Recent advances in laboratory technologies [27–29] 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) [30,31]. Genes associated with N-glycosylation (a subtype of glycosylation where glycans are linked with N-glycosidic bond to asparagine [32]) of human proteins were also shown to be associated with type 1 diabetes, type 2 diabetes and HNF1A-MODY [30,33,34].

N-glycosylation is a ubiquitous co- and post-translational modification that enriches protein structure and function [26,35,36]. Changes in N-glycosylation have been described in different diseases, including type 1 diabetes, type 2 diabetes, gestational diabetes and HNF1A-MODY [37–42], and are thus being considered as biomarkers of ongoing pathological condition. Remarkably, results from several of these studies imply that it is possible to distinguish between different types of diabetes based on N-glycan profiles and to even identify individuals at an increased risk of developing diabetes in the future [38,39,42]. A notable discovery of aberrant N-glycosylation of pancreatic beta cell glucose transporter-2 (Glut-2) in type 2 diabetes that leads to impairment of insulin secretion and can be targeted to suppress diabetes provides a promising new path towards novel drug targets [23,43,44].

It is important to stress that both human plasma and immunoglobulin G (IgG) N-glycomes are stable over time under homeostatic conditions within an individual [45–47], but at the same time remarkably sensitive to different pathological processes [48,49], thus supporting their diagnostic and prognostic potential. Glycosylation changes are well known to influence protein function. For example, the addition of sialic acid converts IgG from proinflammatory, into an anti-inflammatory agent [50]. Furthermore, the addition of core fucose to IgG glycans interferes with IgG binding to FcγRIIIa receptors and decreases its ability to destroy target cells through antibody-dependent cellular cytotoxicity (ADCC) [51]. This feature is currently being exploited in monoclonal antibodies engineering, as lack of core fucose

increases their clinical efficacy through enhancement of ADCC [52].

This review will focus on the scientific discoveries of N-glycosylation changes in type 1 diabetes, type 2 diabetes, gestational diabetes and HNF1A-MODY (Fig. 1, Table 1). Most studies covered in this review are focusing on the N-glycosylation changes in total plasma proteins and IgG, even though N-glycosylation changes in some other proteins in diabetes are increasingly attracting attention [23,44,53,54]. Some of these changes have been related to diabetes onset, and some to diabetes complications. Also, there is accumulating

Table 1. Overview of implicated N-glycans and glycosyltransferase genes with proposed mechanism for the pathogenesis of different types of diabetes.

Type of diabetes	Implicated N-glycans	Implicated proteins/genes	Proposed mechanism	Ref.
Type 1 diabetes	Highly branched N-glycans	T cell proteins, *MGAT5, MGAT1*	Enhanced T cell N-glycan branching through N-glycan–lectin interactions decreases T cell activation and protects against disease (strategy applied: GlcNAc supplementation to nonobese diabetic mouse); joint effects of *MGAT1* haplotypes and other type 1 diabetes risk gene variants were examined and proved significant (*MGAT1* and CTLA-4 joint effects)	[105,168,169]
Type 1 diabetes	Highly branched N-glycans	IL-2Rα (CD25)	Inhibited IL-2Rα N-glycosylation inhibits its surface retention time, its downstream signalling and Th1 cell differentiation; thus, demonstrating dual role of N-glycosylation in T cell activation (strategy applied: glucosamine supplementation)	[73]
Type 1 diabetes	Highly branched N-glycans	Total serum proteins, IgG, α1-acid glycoprotein	Not determined; increase in highly branched N-glycans on these proteins was associated with the disease complications	[40,53]
Type 1 diabetes	α1,2-fucosylated N-glycans	*FUT2*	Causal candidate gene for type 1 diabetes; suggested relationship between host resistance to infections and susceptibility to autoimmune disease through host–pathogen interactions mediated by glycans	[90,92]
Type 2 diabetes	Highly branched N-glycans	Glut-2, *Mgat4a*	High-fat diet reduced expression of transcription factors that inhibited expression of the glycosyltransferase *Mgat4a*; maintenance of glucose transporter by Mgat4a(GnT-IVa)-mediated N-glycosylation preserved glucose transport (strategy applied: high-fat diet)	[23,43,44]
Type 2 diabetes	Highly branched N-glycans	Total plasma proteins	Risk factor; increase in highly branched N-glycans was associated with higher risk of developing this disease	[39]
Type 2 diabetes	Highly branched N-glycans	Total plasma proteins, IgG	Not determined; increase in highly branched N-glycans was associated with type 2 diabetes	[41,58,131]
Type 2 diabetes	N-glycans with α2,6-linked sialic acid (found among highly branched N-glycans)	*ST6GAL1*	Causal candidate gene for type 2 diabetes	[106]
HNF1A-MODY	Antennary fucosylated N-glycans, core fucosylated N-glycans	Total plasma proteins, *HNF1A, FUT3, -5, -6, -8*	Risk factor; it was demonstrated that HNF1A inhibits expression of FUT8 and thus formation of core fucosylated N-glycans and activates fucosyltransferases involved in the formation of antennary fucosylated N-glycans (FUT3, -5, -6) which are decreased upon loss-of-function of *HNF1A*	[30,38,42]
Gestational diabetes	N-glycans with sialic acid, fucose, and mannose	Lactoferrin, secretory IgA	Not determined; alterations were observed during gestational diabetes (comparison between milk from women with vs. women without GDM)	[37]

evidence implicating these changes in mechanism underlying diabetes development (Table 1).

Hexosamine biosynthesis pathway and its role in diabetes through protein N-glycosylation

Glucose-provoked tissue damage is, among other pathways, also assumed to be mediated through the hexosamine biosynthesis pathway (HBP) [55,56]. Under homeostatic conditions, around 3% of total glucose is utilized through this pathway [57]. 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, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc). This was proposed as one of the mechanisms for the observed increase in plasma levels of the highly branched N-glycan structures in type 1 diabetes patients, type 2 diabetes patients and even healthy people at the increased risk of developing diabetes in the future [39,40,58].

Uridine diphosphate *N*-acetylglucosamine, a nucleotide-activated sugar, serves as a substrate for different glycosyltransferases in the process of eukaryotic protein N-glycosylation, which occurs when a block of 14 sugars is relocated cotranslationally to specific asparagine residues in the endoplasmic reticulum [32]. Modifications of resulting N-linked glycans continue in the Golgi complex, where a glycoprotein can undergo a variety of modifications as it is moving towards its final intra- or extracellular location. These modifications can be defined in terms of the quantity of different glycans attached (fucosylation, galactosylation, sialylation, branching) [59] (Fig. 1).

The production of branched N-glycans, and thus the level of branching (biantennary, triantennary and tetraantennary N-glycans), is dependent upon UDP-GlcNAc availability and Golgi enzyme activity [59,60]. All Golgi enzymes responsible for the formation of branched N-glycans, N-acetylglucosaminyltransferases I, II, IV and V (GNT-I, -II, -IV and -V), utilize UDP-GlcNAc as a substrate [61]. The mono-, bi-, tri- and tetra- antennary N-glycans are enzymatic products of GNT-I, -II, -IV and -V respectively (Fig. 1) [26]. GNT-V 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 [62]. GNT-V also catalyses the formation of triantennary N-glycans in the cases when two branches extend from the 1–6 arm of the core, rather than the 1–3 arm (in the latter scenario triantennary N-glycans are formed by the action of GNT-IV) [62].

The GNT-V-branched product is the preferred acceptor, among other branched N-glycans, for subsequent addition of the galectin ligand *N*-acetyllactosamine (LacNAc) [63–65]. The majority of galectins interact with N-glycans at the cell surface, thus forming lattices [66] and increasing glycoprotein cell surface retention time [54,59,67]. There is accumulating evidence for the role of highly branched N-glycans in autoimmunity development, a hallmark of type 1 diabetes. The proposed mechanism involves different glycoproteins present on T cells and alterations of their lectin-N-glycan interactions [54,59,68–73], which is further discussed in the section below.

Ultrasensitivity has been determined within the Golgi pathway branching machinery [59]. Ultrasensitivity represents sharp, switch-like response over a narrow range of stimulus with a characteristic sigmoidal curve [74]. In the terms of Michaelis–Menten kinetics with a characteristic hyperbolic response, ultrasensitivity represents a greater sensitivity to a certain stimulus than the Michaelis–Menten relationship [74]. Affinity for the UDP-GlcNAc decreases stepwise from GNT-I up to GNT-V [59]. GNT-I and GNT-II activities are limited by their affinity for acceptor glycoproteins, whereas GNT-IV and GNT-V are limited by UDP-GlcNAc concentrations [59,60,75]; thus increase in the UDP-GlcNAc results in an ultrasensitive increase in tri- and tetra-antennary N-glycans [59].

It has also been demonstrated that response to increasing hexosamine concentration is dependent upon the number of N-glycans on glycoprotein [59]. Glycoproteins with few N-glycans display switch-like responses, while glycoproteins with more N-glycans display hyperbolic responses; thus, they are differentially regulated by UDP-GlcNAc [59]. The switch-like response has been demonstrated for the glucose transporter and for the cytotoxic T-lymphocyte protein 4 (CTLA-4) [59]; the latter representing an inhibitory glycoprotein in the process of T cell activation, that has been identified as one of the causal candidate genes in type 1 diabetes [76].

A majority of studies in this area focused on UDP-GlcNAc stimulation of O-glycosylation (O-glycosidic linkage between glycan and an amino acid containing a hydroxyl group [77]) when discussing the possible mechanism of diabetic complications as a consequence of increased flux through HBP [78,79]. In a recent study, we have demonstrated that serum N-glycan alterations are associated with diabetic kidney disease [40], which is further discussed below. Taken together, there is accumulating evidence for the role of HBP in both diabetes onset and diabetes complications.

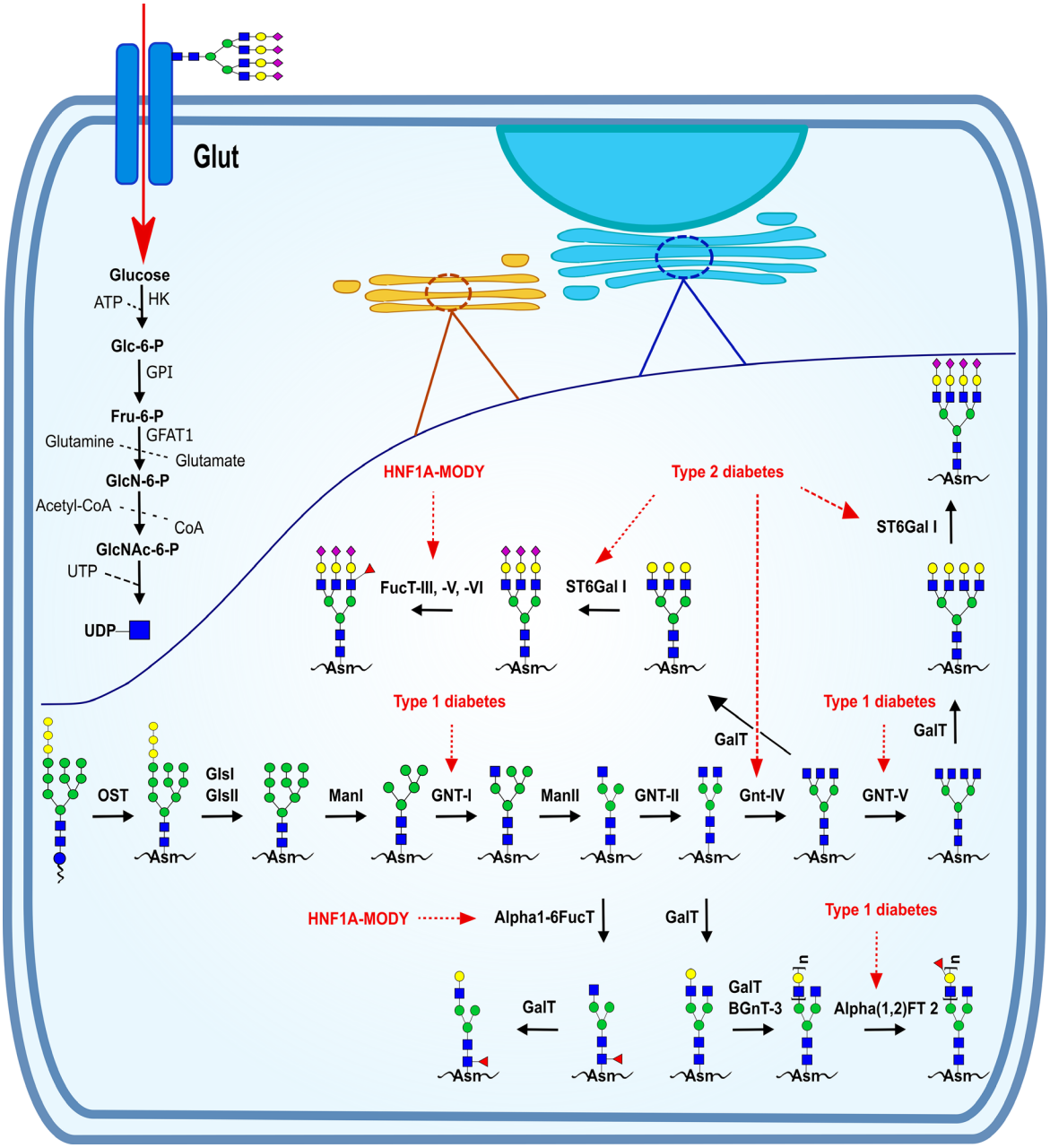

Fig. 1. Schematic representation of hexosamine biosynthesis and N-glycosylation pathways inside the cell with indicated glycosyltransferases that have potential implication in diabetes pathogenesis. Depicted are also N-glycosylated glucose transporter (Glut), nucleus (blue), endoplasmic reticulum (blue) and Golgi complex (brown). The figure was created in Inkscape [170] using glycan structure figures created with GLYCOWORKBENCH software [171] and further processed in Inkscape. Blue squares, green circles, yellow circles, purple diamonds and red triangles represent *N*-acetylglucosamine (GlcNAc), mannose, galactose, *N*-acetylneuraminic acid (sialic acid) and fucose residues respectively. Glut, glucose transporter; HK, hexokinase; GPI, glucose-6-phopshate isomerase; GFAT1, glutamine:fructose-6-phosphate aminotransferase 1; OST, oligosaccharyltransferase; GlsI, glucosidase 1; GlsII, glucosidase 2; ManI, mannosidase 1; GNT-I, alpha-1,3-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyltransferase; ManII, mannosidase 2; GNT-II, alpha-1,6-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyltransferase; Gnt-IV, alpha-1,3-mannosyl-glycoprotein 4-beta-*N*-acetylglucosaminyltransferase; GNT-V, alpha-1,6-mannosyl-glycoprotein 6-beta-*N*-acetylglucosaminyltransferase; GalT, galactosyltransferase; ST6Gal I, beta-galactoside alpha-2,6-sialyltransferase 1; FucT-III, galactoside 3(4)-L-fucosyltransferase; FucT-V, alpha-(1,3)-fucosyltransferase 5; FucT-VI, alpha-(1,3)-fucosyltransferase 6; Alpha1-6FucT, alpha-(1,6)-fucosyltransferase; BGnT-3, *N*-acetyllactosaminide beta-1,3-*N*-acetylglucosaminyltransferase 3; Alpha(1,2)FT 2, galactoside 2-alpha-L-fucosyltransferase 2.

Type 1 diabetes

Type 1 diabetes is an autoimmune disease characterized by T cell (both the $CD4^+$ helper and the $CD8^+$ killer cells)–mediated destruction of the insulin producing pancreatic beta cells and defined by the presence of one or more autoantibodies [8,80,81]. Nevertheless, in the minority of patients with type 1 diabetes there is an absence of pancreatic autoantibodies, and in some patients with clinical diagnosis of type 2 diabetes there is evidence of islet autoimmunity [8,21,82]. It is estimated that the annual increase in the number of children and adolescents diagnosed with type 1 diabetes is around 3% [83,84]. Early diagnosis of type 1 diabetes associates with fewer complications at diagnosis and improved metabolic control in the upcoming years [85]. Association of the disease incidence with geographical latitude has been observed [14,15]. Different incidence rates of type 1 diabetes in two neighbouring populations with no differences in the frequency of type 1 diabetes predisposing genotypes were reported [13]. Type 1 diabetes discordance in monozygotic twins has also been a subject of different studies [86,87]. This all supports the major role of environmental factors in the onset of this worldwide increasing disease and urges their understanding and identification.

FUT2 as one of the causal candidate genes in type 1 diabetes

More than 50 genetic loci have been implicated in type 1 diabetes development [88]. The highest genetic susceptibility is mapped to major histocompatibility complex region [89]. Among the implicated loci, fucosyltransferase 2 gene (*FUT2*) is identified as one of the causal candidate genes [90]. *FUT2* encodes a glycosyltransferase responsible for the addition of fucose α1,2-linked to the terminal galactose on different glycans and thus, the formation of the H antigen in body fluids and on the intestinal mucosa. Individuals homozygous for nonfunctional *FUT2* allele fail to present histo-blood group antigens in saliva and mucosal surfaces (termed as nonsecretors) [91]. Genetic study conducted on type 1 diabetes individuals associated the nonsecretor genotype with the susceptibility to this disease; thus, linking the host resistance to infections with susceptibility to developing autoimmune disease [92].

MGAT5-mediated N-glycan branching impacts T cell activation

There is increasing evidence for the role of highly branched N-glycans in autoimmunity mediated by the *MGAT5* gene [54,68,69]. *MGAT5* encodes the alpha-1,6-mannosyl-glycoprotein 6-beta-*N*-acetylglucosaminyltransferase (GNT-V), an enzyme involved in the synthesis of cell surface ligands for galectins [63–65]. Galectins are family of LacNac-binding lectins found in the extracellular matrix, at the cell surface, and in the cytosol, with at least one conserved carbohydrate-recognition domain [66]. The majority of galectins interact with N-glycans at the surface of the cell, thus forming lattices [66] and increasing glycoprotein retention time at the cell surface [54,59].

Loss of *MGAT5* expression lowers T cell activation threshold due to enhancement of T cell receptors (TCRs) [54]. Clusters of a certain number of TCRs at the antigen presentation site are required for T cell activation [93]. 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 GNT-V-synthesized N-glycans limits TCR recruitment to the site of antigen presentation [54]. *In vivo*, *MGAT5*−/− mice exhibited several autoimmune phenotypes [54]. 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 mannosidase II, an enzyme upstream of GNT-V in the N-glycosylation pathway [70,94].

Sensitivity to autoimmune diseases is affected by differentiation of $CD4^+$ T cells into cytokine-secreting proinflammatory Th1 cells (secrete IFN-γ and TNF-β) or anti-inflammatory Th2 cells (secrete IL-4, IL-5, IL-10, IL-13) [95,96]. Antigen-induced enhancement of the TCR signalling stimulated Th1 and constrained Th2 differentiation [97,98]. It was reported that GNT-V-mediated N-glycosylation negatively regulates Th1 responses and represents an antigen-independent mechanism in Th1 and Th2 regulation [72]. GNT-V -mediated N-glycan branching increased the surface retention time of T cell activation inhibitory glycoprotein CTLA-4 [59], one of the causal candidate genes in type 1 diabetes [76,99].

Nonobese diabetic (NOD) mouse is an animal model of type 1 diabetes [100]. Studies have demonstrated that antibodies against IFN-γ administrated to mice prevented the induction of diabetes [101]. Later studies showed that IFN-γR might play a main role in $CD4^+$ T cell–mediated beta cell damage; however, not in $CD8^+$ T cell mediated [102]. Moreover, IL-4 was shown to prevent insulitis and diabetes development; again, using NOD mice as models, with similar results reported for IL-10 [103,104]. Oral GlcNAc supplementation in these mice enhanced T cell N-glycan branching and protected against the disease [105]. 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 [73]. As previously discussed, the kinetics of GNT-V and number of N-glycans on glycoproteins should be kept in mind, as switch-like sigmoidal responses are characteristic for GNT-V and glycoproteins with few N-glycans upon increase in GlcNAc [59].

N-glycosylation is associated with complications in type 1 diabetes

In a recent study we investigated serum N-glycosylation changes in adult type 1 diabetes patients with kidney disease and demonstrated that N-glycan profile of both total serum proteins and of IgG is altered [40]. The most important observation was increase in complex N-glycans (highly branched, triantennary and tetra-antennary 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 [40]. The most complex IgG N-glycan is a biantennary N-glycan [29], however, again, an increase in more complex (more galactosylated and sialylated biantennary structures) and a decrease in simpler (monogalactosylated biantennary glycans) N-glycans was observed, and correlated with higher HbA1c, higher ACR and greater mean annual decline in eGFR [40]. Glycosylation changes in another plasma protein, α1-acid glycoprotein, have been associated with the vascular complications in type 1 diabetes [53].

Type 2 diabetes

Type 2 diabetes is the most common type of diabetes characterized by relative insulin deficiency, peripheral insulin resistance and thus damaged glucose homeostasis; it is under both genetic and environmental influences [3,106–109]. Most of the individuals with type 2 diabetes are overweight, obese or have an elevated percentage of body fat predominantly in the abdominal region [110,111]. The link of high-fat diet, obesity and diabetes has also been demonstrated on animal models [112,113]. Another common characteristic of this diabetes type is that it remains undiagnosed for many years, especially as hyperglycaemia progresses gradually and has a long presymptomatic phase [4,7]. The risk of developing type 2 diabetes is associated with obesity, age and insufficient physical activity [109]. Loss of glucose-stimulated insulin secretion through the pancreatic beta cell dysfunction and impaired beta cell glucose transporter expression has been implicated in type 2 diabetes pathogenesis [114–116].

N-glycosylation of glucose transporter 2 and novel glycan side-chain biomarkers

Glucose transport system present on the plasma membrane has a role in maintaining the transport of glucose into and out of the cells. Glucose transporters are a family of integral membrane glycoproteins, comprising 13 members [117,118]. Glucose transporter 2 (Glut-2) is a glucose sensor molecule involved in insulin secretion in pancreatic beta cells, glucose transport in kidney and intestine, and glucose delivery into the blood stream through hepatic gluconeogenesis [119]. All vertebrate glucose transporters have a conserved N-glycosylation site situated usually in the first or fifth extracellular loop [120].

Reduced Glut-2 expression at the beta cell surface was observed in mice after high-fat diet [23,116]. Glut-2 murine N-glycosylation was demonstrated to be under both dietary and genetic influence [23]. The retention of Glut-2 on the beta cell surface was mediated by GnT-IVa glycosyltransferase that catalyses the synthesis of a complex Glut-2 N-glycan and thus stabilizes Glut-2 surface retention by enabling lectin-glycan binding. Both dietary and genetic obstruction of this process led to Glut-2 endocytosis, impairment of the insulin secretion and type 2 diabetes pathogenesis [23]. This was later confirmed in human and mouse pancreatic cells; high-fat diet reduced expression of transcription factors, both Foxa2 and Hnf1a, which inhibited *Mgat4a* expression and led to metabolic disorders. Maintenance of glucose transporter expression by GnT-IVa-mediated glycosylation preserved glucose transport [43].

Recently, attention has been drawn to an NMR-derived biomarker, known as GlycA [121–123]. GlycA originates from the N-acetyl methyl groups mostly from *N*-acetylglucosamine residues of glycan branches from acute phase glycoproteins [124] and is considered as a marker of a systemic inflammation [124]. Insulin resistance and beta cell dysfunction is associated with a low-grade chronic systemic inflammation [125]. Increased levels of the GlycA were significantly associated with incident type 2 diabetes in a prospective cohort study [121].

Total plasma protein and IgG N-glycan profiling in type 2 diabetes

Our recent study demonstrated that it is possible to recognize individuals at an increased risk of type 2

diabetes development based on interindividual differences in glycosylation [39]. Total plasma protein N-glycome composition was compared between individuals with registered hyperglycaemia during critical illness, who are known to be at increased risk of future development of type 2 diabetes [126,127], and the individuals who remained normoglycaemic throughout the same condition. Replication cohort comprised individuals with increased levels of HbA1c, incident cases of type 2 diabetes gathered at baseline and controls. Study showed that increased complexity of N-glycan structures (highly branched, galactosylated and sialylated) among total plasma proteins was associated with increased risk of type 2 diabetes development [39]. The same differences were subsequently replicated by the analysis of 820 incident cases of type 2 diabetes in the German part of the EPIC cohort (C. Wittenbecher, T. Pavić, O. Kuxhaus, N. Selak, F. Vučković, J. Štambuk, C. Schiborn, D. Rahelić, S. Dietrich, O. Gornik, H. Boeing, M. Schulze, & G. Lauc, unpublished data). We have reported comparable results in one more study, this time comprising individuals from geographically isolated population [58].

Highly branched sialylated N-glycans have also been shown to increase in other inflammatory diseases [128,129]. Chronic low-grade inflammation is a common feature in subjects with type 2 diabetes [130], which could also indicate that the observed changes are reflection of the initial inflammatory process. It is important to stress out that even though in the previously mentioned study total plasma protein N-glycome that comprises many different glycoproteins was studied, significant differences were observed and enabled identification of individuals at an increased risk of developing this disease [39]. It would be also important to further identify and target specific glycoproteins that are contributing to the observed changes.

In another study comprising individuals with type 2 diabetes authors distinguished between α2,6-linked and α2,3-linked sialylation and demonstrated different effects on sialylation dependent on the linkage. Biantennary a2,6-linked sialylation was increased, and triantennary a2,3-linked sialylation was decreased in individuals with type 2 diabetes in comparison to control group [131]. In this study, authors distinguished between α2,6-linked and α2,3-linked sialylation and demonstrated different effects on sialylation dependent on the linkage. Biantennary α2,6-linked sialylation was increased, and triantennary α2,3-linked sialylation was decreased. Antennary fucose located at the α2,3-sialylated antenna formes terminal sialyl Lewis X (sLeX) epitopes that can be found on different proteins with sialylated fucosylated glycans [132]. Competitive binding and thus inhibition of sLeX/E-selectin interactions has been demonstrated for an acute phase protein, alpha-1-acid glycoprotein (AGP), that also expresses sLeX; blocking of sLeX-dependent immune cell adhesion has been here postulated as an anti-inflammatory property of AGP glycosylation [133]. It is possible, as authors also suggested [131], that reduction of α2,3-linked sialylation observed among individuals with type 2 diabetes could interfere with anti-inflammatory pathways. Increase in biantennary α2,6-linked sialylation was speculated in the study to be derived from immunoglobulin M and haptoglobin and associated with inflammation [131].

Our study of IgG N-glycosylation in type 2 diabetes has shown that a proinflammatory and biologically aged state is reflected on IgG N-glycan profiles [41]. In this study, decrease in galactosylation and sialylation of IgG was observed, as well as increase in IgG fucosylated structures with bisecting GlcNac and decrease in IgG fucosylated structures without bisecting GlcNac. Decrease in IgG galactosylation is associated with a proinflammatory state of IgG [134] and has also been associated with other inflammatory diseases [135,136]. The addition of sialic acid to the terminal end of N-glycan is associated with anti-inflammatory function of the IgG [134]. Core fucose decreases ADCC [51], while the addition of bisecting GlcNAc is speculated to have the opposite effect [137]. It has recently been shown in the mouse model that hyposialylated IgG activates endothelial IgG receptor FcγRIIB and leads to insulin resistance; whereas supplementation with the sialic acid precursor N-acetyl-D-mannosamine restored IgG sialylation and preserved insulin sensitivity [138].

ST6GAL1 as a novel candidate risk gene in type 2 diabetes

Genome-wide association studies published recently in European-descent individuals revealed a novel gene locus implicated in type 2 diabetes, ST6 beta-galactoside alpha-2,6-sialyltransferase 1 gene (*ST6GAL1*) [106]. In individuals of South Asian ancestry, *ST6GAL1* has also been associated with type 2 diabetes [139]. *ST6GAL1* encodes an N-glycosylation pathway protein responsible for the transfer of α2,6-linked sialic acid to galactose-containing substrates [140]. Identified type 2 diabetes risk allele in European-descent individuals was associated with an increase in *ST6GAL1* expression in islets [106]. This finding further corroborates the hypothesis that highly branched N-glycans (which are also highly sialylated) are associated with higher risk of developing type 2 diabetes [39]. The study investigating the specific linkage of the sialic acid, as discussed previously, has demonstrated that total plasma protein

biantennary N-glycans with α2,6-linked sialic acid are increased in type 2 diabetes [131]; further confirming the potential causal relation between α2,6-linked sialic acid and type 2 diabetes. Another study reported that *St6gal1* knockout mice after high-fat diet exhibited increased body weight and visceral adipose tissue weight [141]. This all urges further targeting of *ST6GAL1* in type 2 diabetes.

Other types of diabetes

Maturity-onset diabetes of the young is a monogenic type of diabetes characterized by hyperglycaemia onset at an early age (usually before age of 25, but diagnosis later in life may also occur), damaged insulin secretion and an autosomal dominant pattern of inheritance [8]. The most frequently reported forms are GCK-MODY, HNF1A-MODY and HNF4A-MODY [142]. Mutations in *HNF1A* and *HNF4A* genes cause MODY type 3 and type 1 respectively [143,144]. Many individuals with MODY remain misdiagnosed; classification is even more challenging due to phenotypic overlapping with both type 1 and type 2 diabetes [19,142]. However, since optimal treatments are different, it would be very important to be able to distinguish between MODY and other types of diabetes. Contrary to type 2 diabetes, in HNF1A-MODY and HNF4A-MODY treatment with sulfonylureas is the first-line therapy, since it provides excellent diabetes control for many years [8,145]. Furthermore, since MODY is inherited as an autosomally dominant disease, proper diagnosis may lead to recognition of other affected family members [146].

HNF1A regulates genes in the fucosylation pathway

In the first conducted GWAS on N-glycome data, we demonstrated that *HNF1A* and its downstream target *HNF4A* regulate both fucosyltransferase gene expression and fucose biosynthesis genes. *HNF1A* was found to be both required and sufficient for the expression of multiple genes in the fucosylation pathway, indicating it might be the master regulator of the protein fucosylation [30]. *HNF1A* and *HNF4A* are part of transcriptional regulatory network that controls both liver and pancreas gene expression [147]. Synthesis of fucosylated glycans requires activity of different fucosyltranferases (FUTs), and a nucleotide-activated form of fucose, GDP-fucose, as a substrate during this process [148]. Different important roles for fucosylated glycans have been described, such as roles in blood transfusion reactions, initiation of an inflammatory response, host–microbe interactions, etc. [148–152]. Regulation of transcription activity of the *FUT6* gene has also previously been demonstrated for *HNF4A* [153].

GDP-mannose-4,6-dehydratase (GMDS) is involved in the *de novo* pathway of the GDP-fucose synthesis [148]. Quantitative studies have shown that more than 90% of GDP-fucose comes from *de novo* pathway [154]; the other pathway for synthesis of the GDP-fucose is termed the salvage pathway [148]. Both *HNF4A* and *HNF1A* were shown to regulate the activity of GMDS and L-fucokinase [30], fucose biosynthesis enzymes in both *de novo* and salvage pathway of the GDP-fucose synthesis [148].

In mammals, fucose can be linked to other N-glycans in different linkages; for example, α1,2-linked to the terminal galactose, α1,3- or α1,4-linked to GlcNAc on the outer branch (antennary fucose), or α1,6-linked to the inner GlcNAc binding to protein (core fucose) [155]. For example, FUT1 and -2 are α1,2-fucosyltransferases, FUT3, -4, -5, -6, -7 and -9 are α1,3- or α1,4-fucosyltransferases, and FUT8 is α1,6-fucosyltransferase [155]. Our study demonstrated that *HNF1A* activates several fucosyltransferases involved in the formation of antennary fucosylated N-glycans and inhibits FUT8, which forms core fucosylated N-glycans. These results suggest that by enhancement of antennary FUTs, and downregulation of FUT8, *HNF1A* decreases the consumption of the GDP-fucose for core fucosylation, and thus increases the GDP-fucose availability for antennary fucosylation [30].

It was postulated that the role of transcriptional factors HNF1A and HNF4A might be an essential part of the acute immune response in infection, through their regulation of fucosylation [30]. E-, L- and P-selectins require antennary fucose on their target cell glycoprotein ligands for initiation of inflammation [156]. It was reported for the Leucocyte adhesion deficiency type II, a rare inherited disorder of fucose metabolism, that the lack of fucosylated glycoproteins leads to immunodeficiency caused by the impairment of selectin-mediated leucocyte interactions; which was restored after the oral fucose supplementation [157]. Furthermore, we also showed that fucosylated plasma N-glycans are correlated with acute phase proteins, such as C-reactive protein (CRP) [158].

N-glycans as novel biomarkers of HNF1A-MODY

In a study undertaken soon after the discovery of *HNF1A* regulation of fucosyltransferase and fucose biosynthesis genes, we demonstrated that the proportion of antennary fucosylated N-glycans (DG9-glycan index) provides optimum discrimination between HNF1A-MODY and other types of diabetes [42]. This glycan index was significantly lowered in subjects with

HNF1A-MODY when compared to other diabetes types (type 1, type 2, GCK-MODY) and to controls. However, it did not provide good discrimination between HNF1A and HNF4A-MODY, which was expected as *HNF4A* also regulates fucosylation [30,42]. Since a subset of patients with type 1 diabetes maintain certain endogenous insulin production at the onset of diabetes [159], this glycan biomarker could improve the classification during this period.

The potential of high-sensitivity C-reactive protein (hs-CRP) as a biomarker for HNF1A-MODY has been previously demonstrated; lower levels of hs-CRP were detected in individuals with HNF1A-MODY than in those with other diabetes types [160,161]. We have shown that the DG9-glycan index has comparable power in discriminating HNF1A-MODY from type 2 diabetes; however, it performed better in discrimination from type 1 diabetes. This study also led to the identification of previously unidentified *HNF1A* mutations among diabetes subjects examined, and provided preliminary evidence for the potential of determining the pathogenicity of *HNF1A* variants based on the DG9-glycan index; and thus to evaluate functional significance of novel mutations [42].

Next, we assessed the clinical validity of antennary fucosylated N-glycans and hs-CRP in identifying damaging *HNF1A* alleles in an unselected population of young adults with nonautoimmune diabetes; thus, reflecting a more typical clinical scenario [38]. We have identified novel variants within the *HNF1A* gene and performed the functional assessment of those variants. Both antennary fucosylated N-glycans and hs-CRP enabled differentiation of individuals with damaging *HNF1A* alleles; both biomarkers performed better in selecting subjects for genetic testing than classical clinical criteria or the MODY probability calculator [162]. These results imply that it may be possible to identify individuals with a high risk of having damaging *HNF1A* allele based on glycan biomarkers [38].

HNF1A-MODY is caused by mutations in the *HNF1A* gene, but our recent studies indicated that this gene can also be inactivated epigenetically, with functional consequences on the plasma glycome composition [163]. Some studies reported that a high-fat diet can lead to epigenetic silencing of *HNF1A* [43], indicating that both interindividual variation in *HNF1A* expression and its epigenetic silencing could be causally involved in diabetes development, although is it is currently not possible to know whether this is mediated by changes in protein glycosylation or some other molecular mechanisms. Nevertheless, changes in N-glycome composition could still be used as a biomarker of these early events in diabetes development.

N-glycan profiling in gestational diabetes

Lately, the human milk glycans, comprising both free oligosaccharides (HMOs) and those conjugated to proteins, have started attracting research attention [37,164]. It has been demonstrated that human milk glycoproteins are protective against infective diseases through antimicrobial and immunomodulatory activities [165]. In a study comprising women with gestational diabetes, alterations of human milk lactoferrin and secretory immunoglobulin A N-glycans have been reported, suggesting that dysregulation of glucose during pregnancy might influence innate immune protective functions [37]. The N-glycosylation analyses in gestational diabetes will probably increase in years to come, as it represents a promising new field.

Conclusions and perspectives

Diabetes classification has not been much revised through the last decades [166]. Recently, a new diabetes stratification was proposed, which considers heterogeneity within the type 2 diabetes population and is based on disease progression and risk of diabetic complications [167]. Diabetes classification is not straightforward, and it would be interesting to explore N-glycan potential in this field. For example, there is accumulating evidence for occurrence of a new type of diabetes, termed double diabetes, with classical appearance and clinical diagnosis of type 2 diabetes, but also presence of pancreatic autoantibodies [21,82]. As shown for both HNF1A-MODY and type 2 diabetes [38,39,42], N-glycans have enormous potential as biomarkers that ought to be exploited in the future. This could improve the existing therapeutic strategies, which would bring benefit to both individual patients and the society.

Although there are multiple laboratory tests used to diagnose diabetes, there is a lack of reliable biomarkers that can predict disease progression and development of complications. Therefore, there is a pressing need to find both new screening tools and strategies for preventing development of diabetes and its complications. Studies of N-glycosylation changes have demonstrated their potential for identifying individuals at an increased risk of type 2 diabetes development [39], indicating that interindividual variation in protein glycosylation might be a novel risk factor in diabetes. Furthermore, it has been shown that it is possible to distinguish individuals with different types of diabetes based on their N-glycome data, as it is the case for the HNF1A-MODY and its differentiation from other common and rare types of diabetes [38,42].

N-glycan branching on T cells has been implicated in the development of type 1 diabetes, and N-glycan branching on glucose transporter has been implicated in the development of type 2 diabetes [23,43,44,73,105]. Both discoveries include N-glycan interactions with lectins, which are disrupted upon inhibition of the proper protein N-glycosylation. Findings presented in this review open a whole new field of possible targets in different types of diabetes. Further research is needed to clarify the exact mechanism underlying some of these changes and to develop simpler assays which will enable translating these discoveries to clinical practice.

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Conflict of interest

GL is the founder and CEO of Genos Ltd, a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field. OG is employee of Genos Ltd. NS declared no conflict of interest.

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