# Glycosylation signatures of type 2 diabetes complications and metabolic syndrome

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# CHAPTER 1

## Introduction

## 1.1 General introduction

Metabolic syndrome (MetS) is defined by a clustering of conditions, associated with an increased risk of type 2 diabetes (T2D) and cardiovascular disease (CVD), and it shows predictive power for both [1]. These conditions include high waist circumference, dyslipidemia (high triglycerides and low high density lipoprotein cholesterol), high blood sugar, and high blood pressure [2]. T2D is one of the most prevalent metabolic illnesses globally associated with significant morbidity and mortality due to its complications [3].

High prevalence of T2D and morbidity and mortality due to its complications demonstrate a need for better prevention and treatment. In the current thesis, I investigate whether changes in protein glycosylation are associated with T2D and T2D complications, in order to eventually complement current tests as biomarkers in diagnosis and treatment.

T2D is typically and traditionally diagnosed using clinical tests, such as fasting plasma glucose (FPG), the Oral Glucose Tolerance Test (OGTT), and Hemoglobin A1c (HbA1c). FPG is the blood glucose level after an overnight fast, with a level of 126 mg/dL or higher on two separate occasions being indicative of diabetes. The OGTT measures the blood glucose level before and 2 hours after ingesting a glucose solution, with a level of 200 mg/dL or higher after 2 hours indicating diabetes. HbA1c provides a measure of the average blood glucose level over the past 2-3 months, with a level of 6.5% or higher indicating diabetes [4]. Using just traditional tests like FPG, OGTT and HbA1c, one may miss major variations or key times of hypo- or hyperglycemia [6]. Furthermore, these tests may not detect modest changes in blood sugar levels for prediction of high risk of diabetes [6]. They moreover do not accurately predict diabetes complications in individuals with T2D [7–9]. An early detection of T2D and identification of a predisposition for its development is critical since it may lead to improved prognosis and lifestyle management.
To date, much research has been done on novel markers like adiponectin, fetuin-A, linoleoylglycerophosphocholine (L-GPC), which could identify predispositions for diabetes development, establish an early diagnosis, and predict complications, as well as help monitor the impact of diabetes on body functions, or disease outcomes [6,10–14]. Despite each of these facing its own limitations, this underscores the necessity to investigate newer biomarker. Better prediction can decrease long-range consequences for T2D patients [15,16].

The human plasma *N*-glycome exhibits outstanding potential and, similar to a fingerprint, it is stable within an individual over time [17] but very sensitive to pathophysiological processes; hence, it can reflect the cell conditions at the time of protein secretion [18,19]. This makes it particularly suitable as a biomarker in chronic long term diseases such as T2D.

The qualitative and quantitative examination of protein glycosylation gives a better understanding of their alterations. In addition, recent advancements in glyco-analytical methods

have enabled the implementation of glycomics in diabetes and MetS biomarker research. These efforts explore the possibility of glycans as biomarker sources [20–22].

Development of novel biomarkers can provide a more comprehensive assessment of metabolic syndrome and T2D complications, as well as an approach for simple and cost-effective sample collection. In light of these objectives, this thesis focused on a case-control study to investigate the relationship between *N*-glycosylation and suboptimal health status (SHS) as well as MetS. In addition, released *N*-glycans from plasma proteins and dried blood spots in patients with T2D and its complications were analyzed. Moreover, we developed an automated method to analyze released Immunoglobulin G (IgG) *N*-glycosylation in patients with type 2 diabetic complications in order to investigate *N*-glycans released from one of the most abundant proteins in humans, IgG.

This introduction covers the clinical context of T2D and its complications, as well as MetS and SHS. Additionally, it encompasses the fundamentals of protein glycosylation and discusses the analysis of glycosylation in plasma and dried blood samples using mass spectrometry and ultra-high performance liquid chromatography (UHPLC).

## 1.2 T2D

Diabetes is marked by hyperglycemia (high blood glucose levels). According to the world health organization (WHO), there were 422 million adults with diabetes in 2014, and this figure is expected to rise to 629 million by 2045. Diabetes, with its far-reaching consequences and complications, is a lifetime condition that causes broad socio-economic consequences and confers a reduced life expectancy [10]. About 90-95% of all cases of diabetes is T2D which is characterized by the presence of insulin resistance [23]. T2D may initially be asymptomatic, and vascular complications can already develop in the asymptomatic phase [24]. T2D can develop at any age, even during childhood. However, it is more prevalent among middle-aged and elderly individuals [24]. T2D results from the interaction between genetic, environmental, and behavioral risk factors [25,26].

## 1.3 T2D complications

A large proportion of people with T2D develop complications and some of those complication may already be present at the time of diagnosis. [27,28]These complication have consequences in terms of morbidity and mortality but also for healthcare and economics. Research indicates that diabetes complications significantly impact the overall cost of T2D management, with patients experiencing complications facing costs up to 250% higher than those without complications [29,30] . Various implications including the cost of medication and organ transplantation contribute to

these expenses, but a significant portion of the direct medical cost arises from hospitalization expenses [29,30].

Traditionally, long term diabetes complications have been categorized into microvascular and macrovascular conditions, with resultant retinopathy, nephropathy, neuropathy as microvascular complications, and coronary heart disease, cerebrovascular disease, and peripheral vascular disease as macrovascular complications [31,32]. While T2D is overshadowed by the traditional complications that were mentioned above, different studies described that T2D also directly or indirectly affects other body systems [33,34].

Alongside the aforementioned classification, T2D complications can also be divided into two classes: chronic complications (building up over time) and acute complications (that can happen at any time) [35]. Chronic complications (e.g., retinopathy, foot problems, heart attack/stroke, nephropathy, neuropathy, gum disease, etc.) can develop gradually and can lead to severe damage if they have not been detected and remain untreated. For example, 41.7% of individuals with previously undiagnosed diabetes in the United States developed chronic kidney disease [36,37].

Although T2D is growing increasingly prevalent in children and adolescents across the world, incidence and prevalence rates of complications differ among individuals. Moreover, the reported rates of complications in pediatric and adolescent-onset T2D are commonly detected in adulthood rather than at the original time of diagnosis [38].

- *Cardiovascular disease, Nephropathy, Retinopathy*

One of the significant long-term complications of T2D is CVD, and according to Johns Hopkins Medicine report, it is the most common cause of death for people with diabetes (ca. 75 %) [39]. T2D is an independent cardiovascular risk factor [40]. Persistent high blood sugar shows a strong association with oxidative stress and inflammation which are associated with cardiovascular disease [35,41,42].

Diabetic nephropathy, also called diabetic kidney disease, stands as a common and severe complication of diabetes. Approximately 25% of those afflicted with diabetes will experience the onset of kidney damage [43–45], which can result in kidney failure [43–45]. In total, kidney damage can lead to tissue scarring, urinary protein loss, and eventually chronic kidney disease, which can lead to the need for dialysis or kidney transplantation [46–48]. Currently, the earliest detectable stage of nephropathy is microalbuminuria (MIC), defined as an albumin excretion rate of 30–299 µg/mg of creatinine [49]. The development of nephropathy may be postponed or prevented altogether with early-stage detection and suitable interventions. Microalbuminuria screening is most effective when performed at the time of diabetes diagnosis, and annually thereafter [50]. Thus, in general, better metabolic and blood pressure control and improved management of microalbuminuria are able to slow down disease progression [40].

Another diabetes complication, affecting the eyes, is retinopathy [51]. As the most common microvascular diabetes complication (in more than 40 percent of individuals with diabetes, according to the National Eye Institute [43,52]), it has been used as a general term for all retina disorders caused by diabetes. Non-proliferative and proliferative form are two major types of retinopathy. Moreover, persons with T2D are at a higher risk of other eye problems, such as glaucoma and cataracts [51]. Therefore, there is a strong need to start checking and screening for retinopathy at the earliest stage, right from the time of T2D diagnosis [49,53].

## 1.4 Suboptimal health status (SHS) and metabolic syndrome (MetS)

Suboptimal Health Status (SHS) refers to an overall physical condition between health and illness. It is characterized by the perception of health concerns, chronic tiredness, and a combination of physical symptoms, involving the cardiovascular, digestive, immune and systems. This state must persist for a minimum of three months [54]. In spite of the fact that SHS is also known as a reversible stage of chronic disease [55], a significant part of individuals with non-communicable disorders has a chronic feature by progressing over a couple of years from a reversible suboptimal health state to an irreversible pathological condition, accompanied by associated complications [54,56].

One of the current measurements for SHS is health scales and questionnaires, which can identify reversible damages to health that are difficult to be measured directly. Over the past decades, various types of health scales and questionnaires have been used, like the “Suboptimal Health Status Questionnaire-25 (SHSQ-25)” [54]. This form consists of the five domains [1] fatigue, [2] the cardiovascular system, [3] the digestive tract, [4] the immune system, and [5] mental status [55], and it reports SHS based on its multidimensionality. SHS has been found to be a potential risk factor for chronic conditions like diabetes mellitus, cardiovascular, stroke, and metabolic disorders [54].

MetS is a condition typically diagnosed using a combination of clinical tests and measurements, including waist circumference, blood pressure, fasting glucose level, TG, and HDL cholesterol. Waist circumference is used to assess abdominal obesity, with a measurement of 40 inches or more in men, and 35 inches or more in women, indicative of MetS. Blood pressure is used to assess hypertension, with a measurement of 130/85 mm Hg or higher indicative of MetS. Fasting glucose level is used to assess glucose intolerance, with a level of 100 mg/dL or higher indicative of MetS. TG and HDL cholesterol are used to assess dyslipidemia, with a triglyceride level of 150 mg/dL or higher and an HDL cholesterol level of less than 40 mg/dL in men and less than 50 mg/dL in women indicative of MetS.
It’s important to note that the presence of three or more of these diagnostic criteria is required to diagnose MetS [3,57].

Over the past two decades, the number of people with MetS showed a significant increase worldwide, mainly due to the increasing incidence of obesity and diabetes. Several studies have indicated that although T2D is a heterogeneous disease, MetS can predict future diabetes and CVD [3,57,58], however, it is important to note that MetS's components are all reversible, therefore its diagnosis enables effective treatment, especially body weight control. MetS treatment can reduce the risk of ischemic heart disease and prevent the onset of T2D, if it has not already developed [3,21,57].

## 1.5 Glycosylation

Several types of protein modifications that have been controlled by cells define the final structure, interactions, and functionality of the modified protein. Among different types of modifications, post-translational modifications (PTMs) regulate protein functional activities in cells and interaction with other cellular molecules. PTMs, which change the properties of a protein by proteolytic cleavage and adding a modifying group, include glycosylation, acetylation, methylation, phosphorylation, ubiquitination, nitrosylation, and others [59]. Post-translational modification happens by enzymes, like glycosylation, or through non-enzymatic pathways, such as glycation. In the enzymatic path the enzyme-catalyzed covalent attachment of carbohydrates to a polypeptide is commonly catalyzed by glycosyltransferases, utilizing specific sugar nucleotide donor substrates [2060]. In contrast, through the non-enzymatic glycation process, an aldehyde form of glucose interacts with lysine or arginine residues in proteins and undergoes further alterations that ultimately result in advanced glycation end products that have a substantial role in aging and diseases, particularly diabetes [60]. The relationship between glycosylation and diabetes is relatively understudied [61] and this thesis specifically focuses on the enzymatic pathway, "glycosylation".

Many glycans are found on the outermost surfaces of cellular and secreted macromolecules and are highly diverse [62].

Multiple site preferences for glycan locations on proteins, as well as stereochemical configurations (α or β), vary depending on the connected proteins. In total, there is a theoretical possibility of around $10^{12}$ different branched glycan structures [60].

Protein glycosylation involves several types of modifications: the attachment of *N*-linked glycans, *O*-linked glycans, phosphorylated glycans, glycosaminoglycans, glycosylphosphatidylinositol (GPI) anchors and C-mannosylation of tryptophan residues [60]. *N*-Glycosylation, a prevalent post-translational modification of proteins, is the scope of this thesis.

- *N-Glycan structure*

There are different types of protein glycosylation. Amongst them, 90% of glycoproteins are *N*-glycosylated, and *N*-glycosylation frequently occurs co-translationally, as it is being translated

and transported into the ER and the glycan is attached to the nascent protein. Notably, *N*-glycans play a crucial role in regulating many intracellular and extracellular functions [63,64].

*N*-glycans are covalently bound to the carboxamide nitrogen – therefore the "N" – on asparagine (Asn) residues [63]. In all eukaryotes the *N*-glycan is linked via a beta linkage of an *N*-acetylglucosamine (GlcNAc) to Asn (GlcNAcβ1–Asn). The consensus amino acid motif to receive an *N*-glycan is Asn-X-serine (Ser)/threonine (Thr), where "X" is any amino acid other than proline (Pro). Interestingly, this sequon is found in around 70% of proteins, and approximately 70% of sequons carry an *N*-glycan [65]. The core structure of *N*-glycans that bind to Asn consists of 2* (GlcNAc) and 3*[mannose (Man)], followed by a variable number of other monosaccharides [20]. These can include, for example, Man, GlcNAcs, fucose (Fuc), galactose (Gal), and *N*-acetylneuraminic acid (Neu5Ac), the latter is also known as “sialic acid” (Figure 1) [20] They determine whether the final structure is classified as a high-mannose *N*-glycan, a hybrid *N*-glycan, or a complex *N*-glycan. Biosynthesis of *N*-glycans happens in two phases as well as in two compartments of eukaryotic cells, the endoplasmic reticulum (ER) and the Golgi [63,66]. Furthermore, in this processing pathway, many of the glycosidases and glycosyltransferases are differentially expressed and sensitive to the physiological state of the cell [65].

Endoplasmic reticulum (ER) and Golgi biosynthesize *N*-glycans in a two-step process. Enzymes couple monosaccharides one at a time to the ER's dolichol phosphate to begin the synthesis of the glycan. This process results in the formation of an oligomannose glycan containing nine mannoses and three glucoses on the α1-3 arm. The entire oligomannose glycan is then transferred to the asparagine of the glycosylation site of the protein, and all other glycan structures derive from this singular glycan structure. In the subsequent stage within the Golgi, the glycan continues trimming and rebuilding processes, giving the protein with particular functions. Despite the complexity of this pathway, the glycan intermediates serve an essential function in protein quality control [67].

- *Human protein N-glycosylation*

Glycosylation has a considerable effect on the structural properties of proteins, e.g. conformation, folding, stability, solubility, as well as the biological functions of proteins [68]. It was demonstrated that glycosylation changes happen in various diseases, and they have a high potential to be used as diagnostic and prognostic biomarkers of different diseases and aging [69].

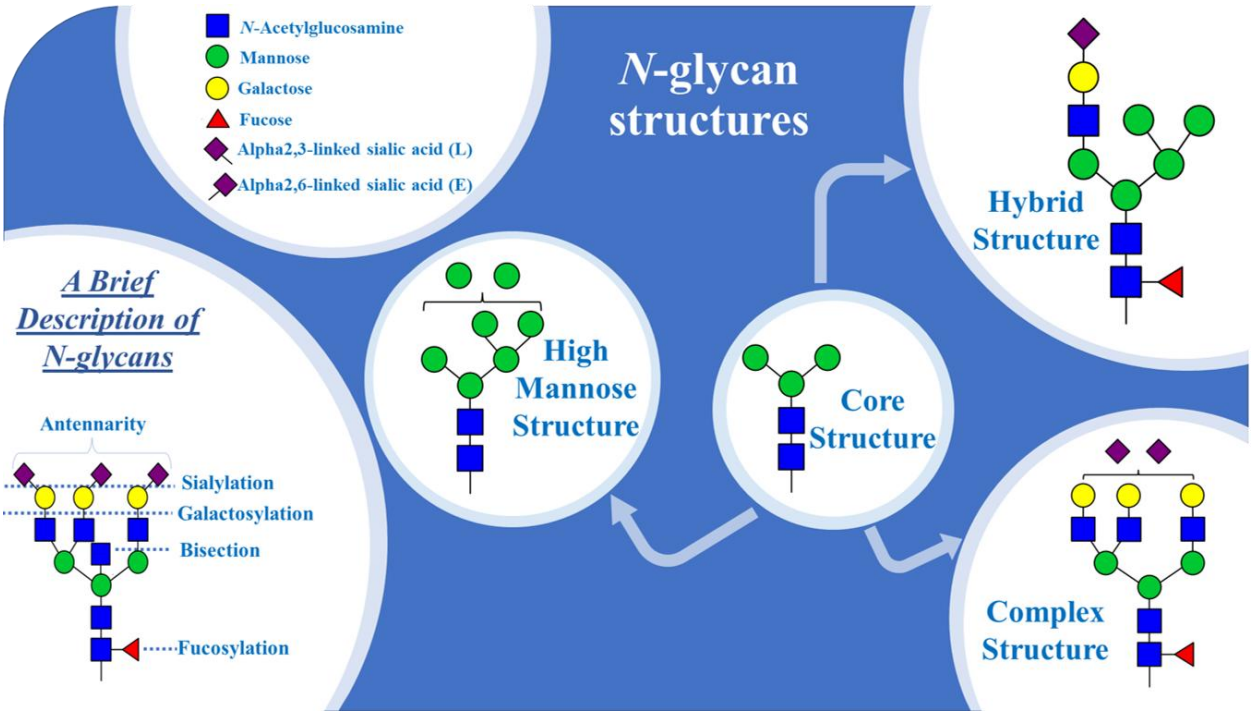


**Figure 1.** Structures of *N*-glycans. The three main types of human *N*-glycans with their core structure, next to the main structural features of complex-type *N*-glycans are displayed. Monosaccharides are introduced in the top left corner. Antennarity (branching): Complex *N*-glycans can have branches initiated by GlcNAc and elongated with Gal-GlcNAc repeats. Sialylation: Sialic acids are commonly found in α-glycosidic linkage to the Carbon-3 or Carbon-6 hydroxyl of galactose.

A large number of studies have been conducted looking for links between *N*-glycosylation of plasma proteins and various chronic disorders. Studies to date have linked changes in *N*-glycosylation to several diseases and conditions such as T2D [69], inflammatory bowel disease [70], various cancers [71], infections [71], and life style factors such as smoking [72].

Proteins present in plasma are differently glycosylated, which is why they contribute uniquely to the overall *N*-glycosylation profile of plasma proteins at the released glycan level [73]. Therefore, a change in glycosylation of an individual protein may be distinctly reflected in the plasma profile. Labeling enzymatically released glycans to allow fluorescence detection (FLD) and to improve ionization for mass spectrometry (MS) is a frequently used approach for *N*-glycan characterization. Using *N*-glycome analysis on whole plasma or serum, one may learn about the levels and glycosylation patterns of main plasma glycoproteins such as immunoglobulins. Moreover, changes in glycosylation of plasma proteins have an impact on their function [73–75].

The current analytical approaches can provide new and useful information in initial research on *N*-glycosylation in various physiological and pathophysiological conditions, but in order to

develop new diagnostic and prognostic markers for target diseases, identifying the leading glycoprotein and analyzing its glycosylation is the path to more sensitive and specific biomarkers. Glycosylation assays of the target glycoprotein in the context of the disease have already been performed for various proteins, of which immunoglobulin G (IgG) is by far the most studied. Changes in IgG glycosylation have been associated with many disorders, some of which have been suggested to have diagnostic and prognostic potential [23,76].

## 1.6 Glycosylation and T2D

Regarding the significance of glycans in the pathophysiology of many diseases, glycoscience play a crucial role in providing further understanding to achieve personalized treatment and disease prognosis [77]. Unlike genetic biomarkers, which remain constant throughout one's lifetime, glycan biomarkers also reflect an individual's physiological and pathological state [78]. The structural diversity of glycans provides different information than proteins, lipids, or nucleic acids. Therefore, glycosylation changes can be used as a tool to diagnose diseases, and/or disease stages which makes them attractive as potential diagnostic/prognostic markers for complex pathologies [79,80].

*N*-glycosylation is associated with the pathogenesis and progression of T2D and is changed in T2D and its complications [69]. Hence, investigating the biomarker potential of *N*-glycosylation in T2D and the role of *N*-glycosylation in the etiology of diabetes is a promising field of biomedical research [10].

Extensive research has been conducted on glycation, uncovering associations in T2D, where the glycation reaction accelerates with hyperglycemic conditions [81,82], and our goal was to further elucidate the role and associations of glycans in T2D. However, glycosylation is a multi-stage enzymatic process regulated by a vast network of genes [80,83]. *N*-glycosylation can reflect the physiological state of an organism and changes thereof. Hence, there is a possibility that *N*-glycans could serve as novel markers that could identify predisposition for diabetes development, establishing early diagnosis, and predicting complications [10].

Altered *N*-glycosylation features that are often reported are increased branching/antennarity (Figure 1) and sialylation of both di- and tri-antennary (Figure 1) glycans in T2D patients. Whether sialic acid is attached to the terminal galactose in α2,6 (“E” in Figure 1) or α2,3 (“L” in Figure 1) linkage is crucial, as variations among isomers can result in differences in biological functionality. A previous study has shown that increased α2,6-sialylation was associated with T2D, while α2,3-sialylation mainly demonstrated a decrease. This might be because of an elevated expression of the ST6GAL1 gene, which encodes the enzyme responsible for attaching sialic acids to glycans in α2,6-linkage and has indeed recently been connected to T2D in a genome-wide association study

[69]. Additionally, aberrant *N*-glycosylation of pancreatic β-cell glucose transporter 2 (GLUT2) in T2D has been reported leading to impairment of insulin secretion [80,84].

## 1.7 Analytical methodology

Discovering the connection of glycan structures with their function, monitoring glycosylation in the diagnosis and prognosis of diseases, and resolving the molecular mechanisms of disease development are important research areas. The development of precise, sensitive and robust methods for glycan analysis is essential. Glycan analysis can be challenging, due to the great diversity of monosaccharide structures, sequences and linkages, and their association with other biomolecules, such as peptides or lipids. Glycans may be characterized in three ways: analyzing intact glycoproteins, analyzing glycopeptides, and by structural analysis of chemically or enzymatically released glycans [85,86].

The focus of this thesis is on the analysis of released glycans from glycoproteins in complex samples (e.g., plasma and blood). Specific structural changes in released glycans such as varying levels of branching, fucosylation, galactosylation, or sialylation can provide helpful information that can help to investigate diseases such as diabetes. If different information on modification of glycoprotein or exact location of glycans on the protein is needed, released glycans cannot supply it. Then working on intact glycoproteins or glycopeptides might be beneficial [20,70].

Different analytical methods for characterization and quantitation of released glycans are available. Nuclear magnetic resonance (NMR) can be used to analyze the structural components of glycans [87,88]. Next to its advantages, like determination of glycan structures and illustrating information on isomers differentiation, it has the limitation that interpretation of NMR data from large structures can be very challenging. Hence, high purity and high sample amount are required compared to more sensitive detection techniques like fluorescence, UV, or mass spectrometry (MS) [89]. The first two are mostly combined with liquid chromatography (LC) and require labeling of glycans. MS, on the other hand, can be coupled with LC via electrospray ionization (ESI) and can be done both with and without prior labeling.

One of the common LC stationary phases to separate glycans is hydrophilic interaction liquid chromatography (HILIC). Because of its high peak capacity, which is defined as the maximum theoretical number of components that can be separated with a resolution of 1 within the specified gradient time, HILIC is effective for complex samples as well as for high sensitivity LC-MS applications. Another separation mode is porous graphitized carbon (PGC), which is an important tool for the chromatography of polar compounds [90–93]. Based on the structure of PGC, it can separate glycan isomers, including linkage isomers. The applicability of PGC may be substantially limited by variations in retention time, which can be attributed to contamination [90]. Reversed-phase (RP) is another technique which usually has high reproducibility and is

extensively available in different laboratories [93,94]. Moreover, glycan separation can be done using capillary gel electrophoresis (CGE), which is mostly combined with fluorescence. CGE is a highly sensitive method that can be used for both qualitative and quantitative analysis. The sample molecules migrate across the gel depending on their size and charge, and are separated based on their relative mobility [95–97].

To date, different analytical techniques, i.e., ultra-high performance liquid chromatography (UPLC) [22], LC-MS [98], matrix-assisted laser desorption/ionization (MALDI)-MS [99], gas chromatography (GC)-MS [100], capillary electrophoresis (CE) [101], etc. have been employed to analyze samples from individuals with diabetes.

In this thesis, UHPLC, and MALDI- time-of-flight (TOF)/ Fourier-transform ion cyclotron resonance (FTICR)-MS were used as analytical techniques for released glycans. These techniques are described in more detail in the following paragraphs, along with the sample preparation approaches used.

### *I. Serum and blood sampling*

In the current thesis projects, *N*-glycans were released from plasma, IgG, or dried blood. In total, there are two types of variation having a significant effect on the result, i.e. technical (such as differences in the blood collection process, sample handling, and storage temperatures) that can cause method variability in the outcome; and biological variation (like age, gender, lifestyle, ethnicity, etc.) [102]. Hence preanalytical processing of samples in a consistent way is very important for the research results and their accuracy.

In this study, glycans were investigated not only in human plasma and IgG but also in DBS. Previous research from our laboratory and others [103,104] demonstrated the stability of glycans in DBSs throughout different drying periods, storage conditions, etc. These enduring glycan profiles provide researchers with the opportunity to collect samples more easily, even allowing patients to perform the collection themselves.

### *II. Glycan release*

Typically, in order to study released *N*-glycans, these structures must be released from the glycoproteins of interest. Intact *N*-glycans can be released chemically or more often, enzymatically, by amidase (peptide N-glycosidase F, PNGase F). One example of the chemical release of glycans is the process of beta-elimination in alkaline conditions. Depending on the alkaline condition, either O-glycans (diluted alkaline) or additionally *N*-glycans (harsh alkaline) can be released from proteins. Even though this process causes damage to the protein backbone and makes GlcNAcs lose their acetyl groups [105].

PNGase F cleaves the link between core GlcNAc and asparagine polypeptide side chain of all *N*-glycan types except those containing α-1,3-fucose bound to mentioned GlcNAc (common in plant and insect glycans) [106].

Depending on the type of glycans that are supposed to be analyzed, it is important to use a suitable enzyme that cleaves specific linkage. Besides that, enzymes shouldn't change glycan structure while it cleaves[107].

Released glycan analysis with the enzymatic process has been used in this thesis. This specific enzyme; the enzyme Peptide: N-glycosidase F; with good selectivity, results in an aspartic acid residue and a glycosylamine [108].

### *III. IgG N-glycosylation analysis*

Glycan changes significantly regulate the function and structure of glycoproteins [109]. One of the outstanding examples is the modulation of immunoglobulin G (IgG). Glycosylation can modulate the functional potential of IgG by defining the structure of the anti-body crystallizable fragment (Fc) region and identifying the Fc receptors to which it can bind [110].

Almost all normal IgG include diantennary *N*-glycans that are bound to an asparagine residue [111], in the heavy chain, the variable regions of the light chain, or both (Figure 2) [112].

Purification of IgG from total plasma protein for glycosylation analysis is often done by affinity for which several types of stationary phases are available.

Moreover, depending on the projects at hand, properties such as high porosity, pH resistance, strong mechanical strength, high surface area, and simple ligand attachment to epoxy groups are critical considerations [113]. Protein G is one of the bioaffinity ligands that is used commonly in the process of isolating IgG [114,115]. Protein G strongly binds to all four subclasses of human IgG through their Fc portion. In chapter four of this thesis, a high throughput method for isolation of IgG *N*-glycans from T2D patient plasma is presented.

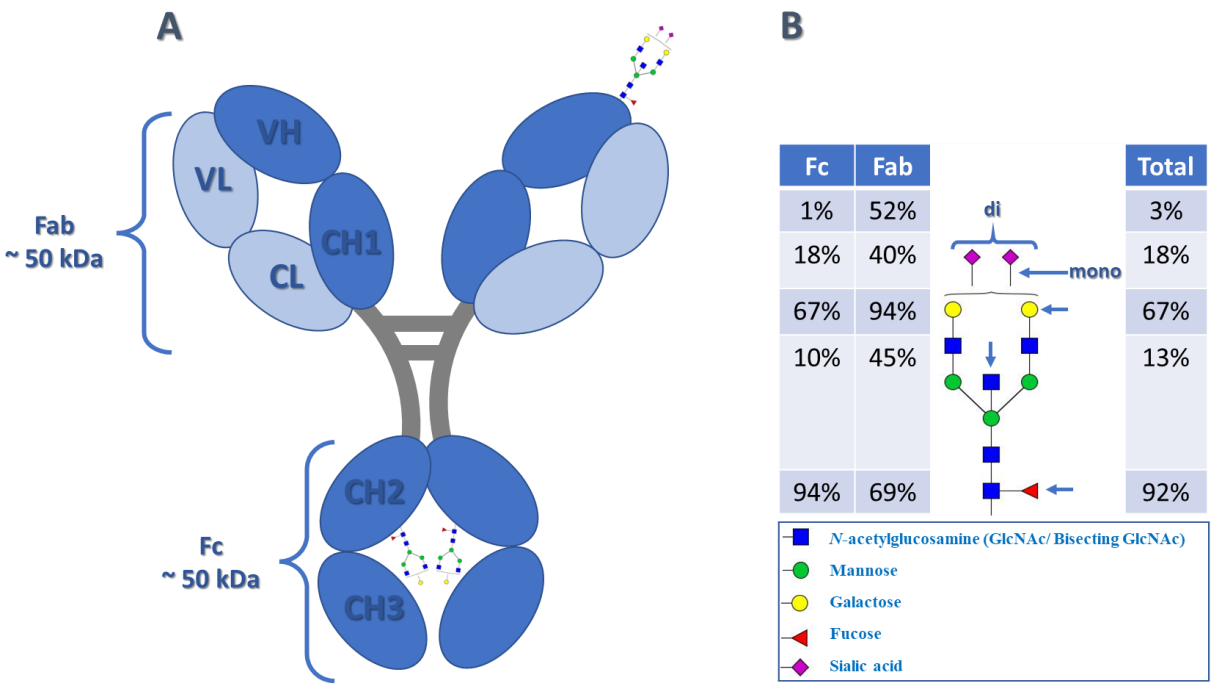


**Figure 2.** Structural scheme of IgG (A) and its *N*-glycosylation (B). Glycans attached to Fc regions are connected to asparagine 297 in the CH2 domain, whereas glycans attached to antigen binding fragment (Fab) regions are attached to *N*-glycosylation sites in the VH and VL domains. The comparison of the glycoprofiles present in Fab and Fc (Abundances in %). *The amino-terminal variable or V domains of the heavy and light chains (VH and VL, respectively); Constant domains or C domains of the heavy and light chains (CH and CL, respectively).* Adapted from [116,117].

## *IV. Glycan derivatization and labeling*

To facilitate detection of glycans in UV and fluorescence, or to improve sensitivity in mass spectrometry (MS), derivatization or fluorescence labeling can be utilized [118–120]. The functional group for derivatization can be a carboxylic acid within sialic acids or hydroxyl groups [121]. In this thesis, the reducing end of the *N*-glycans were modified after their release from the polypeptide backbone.

When using MALDI-MS for the analysis of sialylated glycans, there is a need to stabilize sialic acids which otherwise are lost due to In-source/post-source fragmentation [121–123]. Sialic acids can be methyl- or ethyl esterified [121], as well as amidated and/or permethylated [124].

In *N*-glycans, sialic acids can be linked to their neighboring galactose in either α2,3-linkage or α2,6-linkage. α2,3-Linkage of sialic acids to galactose promotes a lactone formation which is not observed for α2,6-sialylated glycans. Instead, α2,6-linkage will have their carboxylic acid group converted into a methyl or thyl ester (Figure 3).

In this thesis, ethyl esterification was performed to stabilize the sialic acids of total plasma *N*-glycans and IgG *N*-glycans. Using this reaction, in comparison to nonderivatized glycans, mass differences of -18.011 Da and +28.031 Da were induced for the α2,3- and α2,6-linked sialic acids, respectively. And this can be a great advantage of MALDI-MS analysis allowing to obtain information on linkage-specificity of glycans [121].

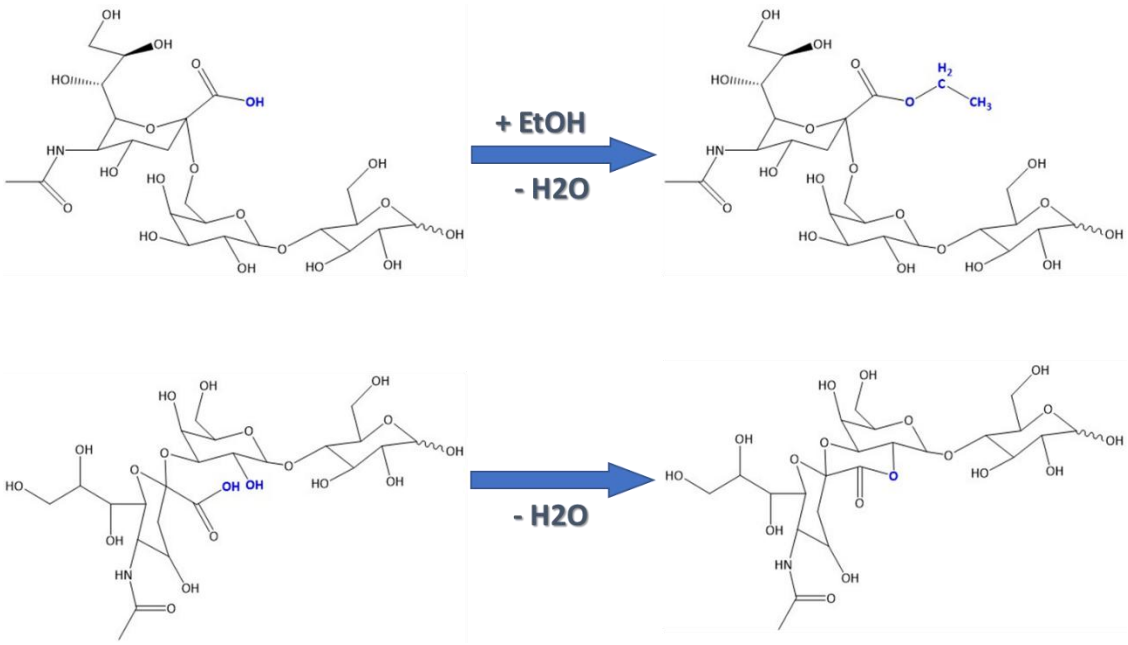


**Figure 3.** Ethyl esterification and lactonization reactions of α2,6-linked and α2,3-linked sialic acids.

## *V. Glycosylation analysis*

The structural characterization of glycans does not only refer to the determination of the composition of monosaccharides and their sequence, but also includes the determination of the stereochemistry of each glycosidic bond and the degree of branching. Among different analytical methods that have been used for *N*-glycan analysis, some of the most commonly used high-throughput methods for more detailed *N*-glycan analysis include hydrophilic interaction liquid chromatography (HILIC) and mass spectrometry (MS).

### a) Hydrophilic interactions liquid chromatography (HILIC) – Ultra High performance liquid chromatography (UHPLC)

High performance liquid chromatography based on hydrophilic interactions is a commonly used technique for glycan separation. Reverse phase chromatography or anion exchange with

fluorescence detection have also been applied, however, liquid chromatography based on hydrophilic interactions is most commonly used to allow improved analyte separation [125,126].

In HILIC analysis, molecules are retained on the column based on their hydrophilic potential, differences in properties that depend on size, charge, composition, structure, glycosidic bonds and degree of glycan branching. The main advantage of this technique is the ability to separate glycan structural isomers, which is difficult to achieve with other methodologies [125].

Glycans are the most commonly labeled with fluorescent labels, such as 2-aminobenzamide (2-AB), procainamide, 2-aminobenzoic acid (2-AA) or 2-aminopyridine (2-AP), which allow the detection of femtomolar amounts of glycans [127]. Of all the fluorescent labels that was mentioned, 2-AB is the most commonly used, as it is compatible with a significant number of glyco-analytical methods, such as mass spectrometry. When labeling glycans with 2-AB, the reaction takes place in a stoichiometric ratio of 1: 1, tagging released glycans at the free reducing end. This allows quantitative measurement of relative amounts of individual glycans during HILIC analysis, as well as the simultaneous separation of neutral and charged glycans [125].

In **Chapters 2**, **4**, **5**, research results are presented from studies using HILIC-UHPLC for the analysis of *N*-glycans that were released from total plasma, IgG, and DBS, respectively.

### b) MALDI Mass spectrometry

Mass spectrometry is often used in glycan analysis because it provides information on the mass and composition of glycans. During MS analysis, glycans may need to be derivatized due to the instability and ionization bias of sialylated glycan species. Therefore, glycans are usually derivatized by reduction and labeling, permethylation, or ethyl esterification of sialic acids [121].

MALDI and electrospray ionization (ESI) are the two main ionization approaches that are used for glycan analysis. MALDI-MS is used for rapid glycan profiling, often with a TOF analyzer or FTICR, for the purpose of obtaining a glycan profile and for detecting exoglycosidase digestion products in a manner similar to UPLC analysis [128,129].

MALDI-MS is often the first technique used in glycan analysis, as it is an experiment with low sample consumption, and can be used to assess the variety of glycans present in the sample. Neutral glycans can be better detected in the positive ionization mode, usually as $[M + Na]^+$ adducts. As it was mentioned, because sialylated glycans are labile during ionization, sialylated glycans are rather analyzed in negative ionization mode or derivatized prior to analysis.

In two of the studies contained in this thesis, Chapters 3 and 4, *N*-glycans released from total plasma and IgG were analyzed using MALDI-TOF/FTICR-MS.

## 1.8 Scope

Investigating new biomarkers and improving our understanding of the pathophysiology of MetS, T2D and its complications are the overarching goals of this thesis. For this objective, the *N*-glycan profile of plasma and DBS in patients with MetS and T2D and the compatibility of these data have been studied using human samples.

In **Chapter 1** of this thesis, an overview of MetS and T2D with a focus on associated complications is presented, in addition to an overview of glycosylation and techniques for glycan analysis.

*N*-glycosylation has already been studied in various Asian and European communities. However, there is a notable lack of such studies among geographically and genetically isolated populations. Therefore, **Chapter 2** aims to characterize the plasma *N*-glycome in both healthy individuals and those with SHS and MetS within the Ghanaian population in West Africa. Furthermore, **Chapter 2** includes a comparison of the Ghanaian findings with previously examined results reported for Chinese Han, Croatian, and Orcadian populations.

While the relevance of glycosylation in T2D is known, the association between TPNG and T2D complications like retinopathy, nephropathy, and CVD remains poorly explored. **Chapter 3** addresses these shortcomings by investigating TPNG in relation to complications in T2D within subsamples of two independent Dutch cohorts. This exploration utilizes an advanced, high-resolution analytical technique, MALDI-TOF/FTICR-MS, providing the TPNG in prevalent and incident retinopathy, nephropathy, and CVD, factors that play a significant role in influencing the health challenges associated with T2D.

As previously mentioned, **Chapter 3** explores the associations between the *N*-glycosylation of total plasma proteins and complications associated with T2D. Research indicates that the *N*-glycosylation of IgG contributes to the function of IgG in inflammation. It has been demonstrated that inflammation plays a crucial role in the development of T2D complications. To gain deeper insights into the pathophysiology of diabetes complications and to develop biomarkers for T2D complications, **Chapter 4** presents the associations between IgG *N*-glycosylation and both prevalent and incident cases of diabetic nephropathy, retinopathy, and macrovascular complications. The study employs high-throughput techniques, UPLC and MALDI-FTICR-MS across three extensive European cohorts.

A less invasive alternative to traditional venous blood sampling is needed, particularly for patients facing challenges such as travel restrictions. The use of DBS is explored instead of plasma, addressing issues like extended storage in biobanks or limitations in shipping liquid samples. On the other hand, for individuals, such as those with pre-diabetes or diabetes, a self-sampling protocol proves beneficial. However, utilizing already stored samples to make DBS may not always be feasible if they were not initially intended for that purpose. **Chapter 5** introduces

an improved method, similar to the one employed in **Chapter 2**, for analyzing DBS *N*-glycans. It focuses on different preparation approaches, particularly cases where only frozen blood or separated frozen-blood cells and corresponding frozen plasma are available. The method is also used to compare DBS *N*-glycans from pre-diabetic and diabetic subjects, derived from separated frozen-blood cells and corresponding frozen plasma samples that were stored for an extended period.

In **Chapter 6**, the association of vascular complications with T2D and the link with *N*-glycan biomarkers is discussed, next to *N*-glycan biomarkers of MetS. Furthermore, the chapter discusses the potential applications of our research including DBS glycomics for future diagnostic and prognostic purposes.

## References


1. Cornier, M. A. *et al.* The metabolic syndrome. *Endocr Rev* **29**, 777–822 (2008).

2. Paulhus, M. & Salvo, M. C. Metabolic syndrome: Risk factors and recommendations. *U.S. Pharmacist* **39**, 51–54 (2014).

3. Sharma, S. R. *et al.* Social determinants of common metabolic risk factors (high blood pressure, high blood sugar, high body mass index and high waist-hip ratio) of major non-communicable diseases in South Asia region: A systematic review protocol. *Syst Rev* **6**, 183 (2017).

4. Gholap, N. N., Davies, M. J., Mostafa, S. A. & Khunti, K. Diagnosing type 2 diabetes and identifying high-risk individuals using the new glycated haemoglobin (HbA1c) criteria. *British Journal of General Practice* **63**, (2013).

5. Chatterjee, S., Khunti, K. & Davies, M. J. Type 2 diabetes. *The Lancet* **389**, 2239–2251 (2017).

6. Dorcely, B. *et al.* Novel biomarkers for prediabetes, diabetes, and associated complications. *Diabetes, Metabolic Syndrome and Obesity* **10**, 345–361 (2017).

7. Lim, W. Y. *et al.* Screening for diabetes with HbA1c: Test performance of HbA1c compared to fasting plasma glucose among Chinese, Malay and Indian community residents in Singapore. *Sci Rep* **8**, 12419 (2018).

8. Doumatey, A. P. *et al.* Serum fructosamine and glycemic status in the presence of the sickle cell mutation. *Diabetes Res Clin Pract* **177**, 108918 (2021).

9. Agiostratidou, G. *et al.* Standardizing clinically meaningful outcome measures beyond HbA1c for type 1 diabetes: A consensus report of the American Association of Clinical Endocrinologists, the American Association of Diabetes Educators, the American Diabetes Association, the Endocrine Society, JDRF International, The Leona M. and Harry B. Helmsley Charitable Trust, the Pediatric Endocrine Society, and the T1D Exchange. *Diabetes Care* **40**, 1622–1630 (2017).

10. Štambuk, T. & Lauc, G. N-Glycans and Diabetes. in *Encyclopedia of Cell Biology, Second Edition* (eds. Bradshaw Ralph A., Hart Gerald W. & Stahl Philip D.) 506–515 (2023). doi:10.1016/B978-0-12-821618-7.00001-8.

11. Jiang, Y., Owei, I., Wan, J., Ebenibo, S. & Dagogo-Jack, S. Adiponectin levels predict prediabetes risk: the Pathobiology of Prediabetes in A Biracial Cohort (POP-ABC) study. *BMJ Open Diabetes Res Care* **4**, 194 (2016).

12. Gall, W. E. *et al.* α-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS One* **5**, (2010).

13. Stefan, N. *et al.* Impact of the adipokine adiponectin and the hepatokine fetuin-A on the development of type 2 diabetes: Prospective cohort- and cross-sectional phenotyping studies. *PLoS One* **9**, (2014).

14. Cobb, J. *et al.* A Novel Fasting Blood Test for Insulin Resistance and Prediabetes. *J Diabetes Sci Technol* **7**, (2013).

15. Deberneh, H. M. & Kim, I. Prediction of type 2 diabetes based on machine learning algorithm. *Int J Environ Res Public Health* **18**, 3317 (2021).

16. Grundy, S. M. *et al.* Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute scientific statement. *Circulation* **112**, 2735–2752 (2005).

17. Gornik, O. *et al.* Stability of N-glycan profiles in human plasma. *Glycobiology* **19**, 1547–1553 (2009).

18. Drake, P. M. *et al.* Sweetening the pot: Adding glycosylation to the biomarker discovery equation. *Clin Chem* **56**, 223–236 (2010).

19. Keser, T. *et al.* Increased plasma N-glycome complexity is associated with higher risk of type 2 diabetes. *Diabetologia* **60**, 2352–2360 (2017).

20. Vreeker, G. C. M. GLYCOSYLATION PROFILING WITH MASS SPECTROMETRY method development and application to cancer biomarker studies. (2021).

21. Adua, E. *et al.* Utilization of N-glycosylation profiles as risk stratification biomarkers for suboptimal health status and metabolic syndrome in a Ghanaian population. *Biomark Med* **13**, 1273–1287 (2019).

22. Adua, E. *et al.* N-glycosylation profiling of Type 2 diabetes mellitus from baseline to follow-up: An observational study in a Ghanaian population. *Biomark Med* **15**, 467–480 (2021).

23. Tijardović, M. HIGH-THROUGHPUT N-GLYCOSYLATION ANALYSIS OF ALPHA-1-ACID GLYCOPROTEIN IN THE IDENTIFICATION OF DIABETES MELLITUS BIOMARKERS. (Zagreb, 2022).

24. Tuomi, T. Type 1 and Type 2 Diabetes What Do They Have in Common? *Diabetes* **54(suppl_2)**, S40–S45 (2005).

25. Chen, L., Dianna J. Magliano & Paul Z. Zimmet. The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives. *Nat Rev Endocrinol* **8**, 228–236 (2012).

26. Olokoba, A. B., Obateru, O. A. & Olokoba, L. B. Type 2 diabetes mellitus: A review of current trends. *Oman Med J* **27**, 269–273 (2012).

27. An, J. *et al.* Prevalence and incidence of microvascular and macrovascular complications over 15 years among patients with incident type 2 diabetes. *BMJ Open Diabetes Res Care* **9**, e001847 (2021).

28. Liu, Z., Fu, C., Wang, W. & Xu, B. Prevalence of chronic complications of type 2 diabetes mellitus in outpatients-a cross-sectional hospital based survey in urban China. *Health Qual Life Outcomes* **8**, 62 (2010).

29. Liebl, A., Khunti, K., Orozco-Beltran, D. & Yale, J. F. Health economic evaluation of type 2 diabetes mellitus: A clinical practice focused review. *Clin Med Insights Endocrinol Diabetes* **8**, 13–19 (2015).

30. Williams, R., Gaaf, L. Van, Lucionp, C. & Wiliiams, R. Assessing the impact of complications on the costs of Type II diabetes. *Diabetologia* **45**, 13–17 (2002).

31. Cho Nam Han *et al. International Diabetes Federation. IDF Diabetes Atlas (Seventh Edition)*. (Brussels, Belgium: International Diabetes Federation, 2015).

32. DM, N. Long-term complications of diabetes mellitus. *N Engl J Med* **328**, 1676–85 (1993).

33. Lu FP, Lin KP & Kuo HK. Diabetes and the risk of multi-system aging phenotypes: a systematic review and meta-analysis. *PLoS One* **4**, e4144 (2009).

34. Gregg, E. W., Sattar, N. & Ali, M. K. The changing face of diabetes complications. *Lancet Diabetes Endocrinol* **4**, 537–547 (2016).

35. Geddes, W. S. Complications of diabetes. *South Med J* **19**, 248–253 (1926).

36. Plantinga LC, Crews DC, Coresh J, Miller ER, S., R, Yee J, Hedgeman E, Pavkov M, E. M. & Al., W. D. et. Prevalence of chronic kidney disease in US adults with undiagnosed diabetes or prediabetes. *Clin J Am Soc Nephrol* **5**, 673–682 (2010).

37. Rudman, N., Gornik, O. & Lauc, G. Altered N-glycosylation profiles as potential biomarkers and drug targets in diabetes. *FEBS Lett* **593**, 1598–1615 (2019).

38. Jordan, D. N. & Jordan, J. L. Pediatric type 2 diabetes mellitus complications: A systematic review of the literature. *J Diabetes Res Clin Metab* **1**, 24 (2012).

39. O'Gara PT *et al.* 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* **61**, e78–e140 (2013).

40. Schlienger, J. L. Complications du diabète de type 2. *Presse Medicale* **42**, 839–848 (2013).

41. Oguntibeju, O. O. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. *Int J Physiol Pathophysiol Pharmacol* **11**, 45–63 (2019).

42. Pashkow, F. J. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? *Int J Inflam* **2011**, 1–9 (2011).

43. Papatheodorou, K., Banach, M., Bekiari, E., Rizzo, M. & Edmonds, M. Complications of Diabetes 2017. *J Diabetes Res* **2018**, (2018).

44. Gnudi, L. & Long, D. A. *Diabetic Nephropathy: An Overview (Eds) Diabetic Nephropathy. Methods in Molecular Biology*. vol. 2067 (Humana, New York, NY., 2020).

45. Gross, J. L. *et al.* Diabetic Nephropathy: Diagnosis, Prevention, and Treatment. *Diabetes Care* **28**, 164–176 (2005).

46. Wing, E. J. & Schiffman, F. *Cecil Essentials of Medicine (10th Edition)*. (Pennsylvania: Elsevier, 2022).

47. Saedi E, Gheini MR, Faiz F & Arami MA. Diabetes mellitus and cognitive impairments. *World J Diabetes* **7**, 412–422 (2016).

48. Kitabchi AE, Umpierrez GE, Miles JM & Fisher JN. Hyperglycemic crises in adult patients with diabetes. *Diabetes Care* **32**, 1335–1343 (2009).

49. Amutha, A. & Mohan, V. Diabetes complications in childhood and adolescent onset type 2 diabetes - A review. *J Diabetes Complications* **30**, 951–957 (2016).

50. American Diabetes Association. 'Standards of medical care in diabetes—2014.' *Diabetes Care* **37**, S14–S80 (2014).

51. Barrett, T. G. Complications of type 2 diabetes. *Annales Nestle* **63**, 17–25 (2005).

52. Fong, D. S. *et al.* Diabetic Retinopathy. *Diabetes Care* **26 (suppl_1)**, s99–s102 (2003).

53. Eppens, M. C. *et al.* Prevalence of diabetes complications in adolescents with type 2 compared with type 1 diabetes. *Diabetes Care* **29**, 1300–1306 (2006).

54. Wang, W. *et al.* All around suboptimal health — a joint position paper of the Suboptimal Health Study Consortium and European Association for Predictive , Preventive and Personalised Medicine Traditional Chinese medicine. *EPMA Journal* **12**, 403–433 (2021).

55. Ge, S. *et al.* Suboptimal health status as an independent risk factor for type 2 diabetes mellitus in a community-based cohort : the China suboptimal health cohort study. *EPMA Journal* **10**, 65–72 (2019).

56. O., G. *Flammer Syndrome – from Phenotype to Associated Pathologies, Prediction, Prevention and Personalisation (1st Edition)*. *Springer, Cham* vol. 11 (Springer Cham, 2019).

57. Wilson, P. W. F., D'Agostino, R. B., Parise, H., Sullivan, L. & Meigs, J. B. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation* **112**, 3066–3072 (2005).

58. Neuhauser, H. K. The metabolic syndrome. *Lancet* **366**, 1415–1428 (2005).

59. Qausain, S., Srinivasan, H., Jamal, S., Nasiruddin, M. & Alam Khan, Md. K. Phosphorylation and Acetylation of Proteins as Posttranslational Modification: Implications in Human Health and Associated Diseases (Chapter 3). in *Protein Modificomics* (eds. Ali Dar, T. & Singh, L. R.) 69–86 (Academic Press, 2019). doi:10.1016/B978-0-12-811913-6.00003-5.

60. Schjoldager, K. T., Narimatsu, Y., Joshi, H. J. & Clausen, H. Global view of human protein glycosylation pathways and functions. *Nat Rev Mol Cell Biol* **21**, 729–749 (2020).

61. Dotz, V. *et al.* Plasma protein N-glycan signatures of type 2 diabetes. *Biochim Biophys Acta Gen Subj* **1862**, 2613–2622 (2018).

62. Moremen, K. W., Tiemeyer, M. & Nairn, A. V. Vertebrate protein glycosylation: Diversity, synthesis and function. *Nat Rev Mol Cell Biol* **13**, 448–462 (2012).

63. Reily, C., Stewart, T. J., Renfrow, M. B. & Novak, J. Glycosylation in health and disease. *Nat Rev Nephrol* **15**, 346–366 (2019).

64. Trombetta ES. The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. *Glycobiology* **13**, 77R-91R (2003).

65. Stanley P., Taniguchi N. & Aebi M. N-Glycans (Chapter 9) (3rd edition). in *Essentials of Glycobiology* (eds. Varki A, Cummings RD, Esko JD & et al.) (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2017). doi:10.1101/glycobiology.3e.009.

66. Colley K.J., Varki A. & Kinoshita T. Cellular Organization of Glycosylation (Chapter 4) (3rd edition). in *Essentials of Glycobiology* (eds. Varki A, Cummings RD, Esko JD & et al.) (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2017). doi:10.1101/glycobiology.3e.004.

67. Helenius, A. & Aebi, M. Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**, 1019–1049 (2004).

68. Krištić, J. Changes of immunoglobulin G glycosylation with age. (Zagreb University, 2018).

69. Dotz, V. & Wuhrer, M. N-glycome signatures in human plasma: associations with physiology and major diseases. *FEBS Lett* **593**, 2966–2976 (2019).

70. Clerc, F. *et al.* Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. *Gastroenterology* **155**, 829–843 (2018).

71. Zhang W., Yang Z., Gao X. & Wu Q. Advances in the discovery of novel biomarkers for cancer: spotlight on protein N-glycosylation. *Biomark Med* **14**, 1031–1045 (2020).

72. Knežević, A. *et al.* Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. *Glycobiology* **20**, 959–969 (2010).

73. Clerc, F. *et al.* Human plasma protein N-glycosylation. *Glycoconj J* **33**, 309–343 (2016).

74. Dalziel, M., Crispin, M., Scanlan, C. N., Zitzmann, N. & Dwek, R. A. Emerging principles for the therapeutic exploitation of glycosylation. *Science (1979)* **343**, 1235681 (2014).

75. Murphy, K., Travers, P., Walport, M. & Janeway, C. *Janeway's Immunobiology*. vol. 1 (Garland Science, 2011).

76. Gudelj, I., Lauc, G. & Pezer, M. Immunoglobulin G glycosylation in aging and diseases. *Cell Immunol* **333**, 65–79 (2018).

77. *National Research Council (US) Committee on Assessing the Importance and Impact of Glycomics and Glycosciences. Transforming Glycoscience: A Roadmap for the Future*. (Washington (DC): National Academies Press (US), 2012). doi:10.17226/13446.

78. J, A. The impact of glycobiology on medicine. *Trends Immunol* **22**, 237–239 (2001).

79. Singh, S. S. *et al.* Association of the IgG N -glycome with the course of kidney function in type 2 diabetes. *BMJ Open Diabetes Res Care* **8**, 1–7 (2020).

80. Štambuk, T. & Gornik, O. Protein glycosylation in diabetes. in *The Role of Glycosylation in Health and Disease. Advances in Experimental Medicine and Biology* (eds. Lauc, G. & Trbojević-Akmačić, I.) vol. 1325 285–305 (2021).

81. Ishrat, N. *et al.* Role of Glycation in Type 2 Diabetes Mellitus and Its Prevention through Nymphaea Species. *Biomed Res Int* **2021**, (2021).

82. Ahmad, S. *et al.* Glycoxidation of biological macromolecules: a critical approach to halt the menace of glycation. *Glycobiology* **24**, 979–990 (2014).

83. Ulrich P, C. A. Protein glycation, diabetes, and aging. *Recent Prog Horm Res* **56**, 1– 21 (2001).

84. Ohtsubo K, Takamatsu S, Gao C, et al. N-glycosylation modulates the membrane sub-domain distribution and activity of glucose transporter 2 in pancreatic beta cells. *Biochem Biophys Res Commun* **434**, 346–351 (2013).

85. Marino, K., Bones, J., Kattla, J. J. & Rudd, P. M. Nature Chemical Biology. *Nat Chem Biol* **6**, 713–723 (2010).

86. Pavić, T. GLYCOSYLATION OF PLASMA PROTEINS IN HNF1A–MATURITY ONSET DIABETES OF THE YOUNG. (Zagreb University, 2017).

87. Battistel, M. D.; Azurmendi, H. F.; Yu, B.; Freedberg, D. I. NMR of Glycans: Shedding New Light on Old Problems. *Prog. Nucl. Magn. Reson. Spectrosc.* **79**, 48–68 (2014).

88. Lundborg, M. & Widmalm, G. Structural Analysis of Glycans by NMR Chemical Shift Prediction. *Anal. Chem.* **83**, 1514–1517 (2011).

89. Taylor, M. E. & Drickamer, K. *Introduction to Glycobiology (Third Edition)*. (Oxford University Press: Oxford, 2011).

90. Bapiro, T. E., Richards, F. M. & Jodrell, D. I. Understanding the Complexity of Porous Graphitic Carbon (PGC) Chromatography: Modulation of Mobile-Stationary Phase Interactions Overcomes Loss of Retention and Reduces Variability. *Anal Chem* **88**, 6190–6194 (2016).

91. Wuhrer, M., De Boer, A. R. & Deelder, A. M. Structural Glycomics Using Hydrophilic Interaction Chromatography (HILIC) with Mass Spectrometry. *Mass Spectrom Rev* **28**, 192–206 (2009).

92. Ruhaak, L. R., Deelder, A. M. & Wuhrer, M. Oligosaccharide Analysis by Graphitized Carbon Liquid Chromatography-Mass Spectrometry. *Anal Bioanal Chem* **394**, 163–174 (2009).

93. Vreeker, G. C. M. & Wuhrer, M. Reversed-phase separation methods for glycan analysis. *Anal Bioanal Chem* **409**, 359–378 (2017).

94. Melmer, M., Stangler, T., Premstaller, A. & Lindner, W. Comparison of Hydrophilic-Interaction, Reversed-Phase and Porous Graphitic Carbon Chromatography for Glycan Analysis. *Journal of chromatography A* **1218**, 118–123 (2011).

95. Lageveen-Kammeijer, G. S. M. *et al.* Highly Sensitive CE-ESI-MS Analysis of N-Glycans from Complex Biological Samples. *Nat Commun* **10**, 2137 (2019).

96. Szabo, Z., Guttman, A., Rejtar, T. & Karger, B. L. Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. *Electrophoresis* **31**, 1389–1395 (2010).

97. Harstad, R. K., Johnson, A. C., Weisenberger, M. M. & Bowser, M. T. Capillary Electrophoresis. *Anal Chem* **88**, 299–319 (2016).

98. Zhang, M., Xu, W. & Deng, Y. A new strategy for early diagnosis of type 2 diabetes by standard-free, label-free LC-MS/MS quantification of glycated peptides. *Diabetes* **62**, 3936–3942 (2013).

99. Memarian, E. *et al.* Plasma protein N- glycosylation is associated with cardiovascular disease, nephropathy, and retinopathy in type 2 diabetes . *BMJ Open Diabetes Res Care* **9**, e002345 (2021).

100. Yan, Y. *et al.* Discovery of potential biomarkers in exhaled breath for diagnosis of type 2 diabetes mellitus based on GC-MS with metabolomics. *RSC Adv* **4**, 25430–25439 (2014).

101. Torok, R. *et al.* N-glycosylation profiling of human blood in type 2 diabetes by capillary electrophoresis: A preliminary study. *Molecules* **26**, 6399 (2021).

102. Lista, S., Faltraco, F. & Hampel, H. Biological and Methodical Challenges of Blood-Based Proteomics in the Field of Neurological Research. *Prog Neurobiol* **101**, 18–34 (2013).

103. Ruhaak, L. R., Miyamoto, S., Kelly, K. & Lebrilla, C. B. N-Glycan Profiling of Dried Blood Spots. *Anal Chem* **84**, 396–402 (2012).

104. Vreeker, G. C. M. *et al.* Dried blood spot N-glycome analysis by MALDI mass spectrometry. *Talanta* **205**, 120104 (2019).

105. Mulloy B., Dell A., Stanley P. & et al. Structural Analysis of Glycans (Chapter 50) (3rd edition). in *Essentials of Glycobiology* (eds. Varki A., Cummings R.D., Esko J.D. & et al.) (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2017). doi:10.1101/glycobiology.3e.050.

106. TRETTER, V., ALTMANN, F. & MÄRZ, L. Peptide-N4-(N-acetyl-β-glucosaminyl) asparagine amidase F cannot release glycans with fucose attached α1 → 3 to the asparagine-linked N-acetylglucosamine residue. *European Journal of Biochemistry* **199**, 647–652 (1991).

107. Merry, T. & Astrautsova, S. Chemical and enzymatic release of glycans from glycoproteins. *Methods Mol Biol* **213**, 27–40 (2003).

108. Rudd, P., Karlsson, N. G., Khoo, K. H. & Packer, N. H. Glycomics and Glycoproteomics (Chapter 51) (3rd edition). in *Essentials of Glycobiology* (eds. Varki A., Cummings R.D., Esko J.D. & et al.) (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2017). doi:10.1101/glycobiology.3e.051.

109. Skropeta, D. The effect of individual N-glycans on enzyme activity. *Bioorg. Med. Chem.* **17**, 2645–2653 (2009).

110. Jennewein, M. F. & Alter, G. The Immunoregulatory Roles of Antibody Glycosylation. *Trends Immunol* **38**, 358–372 (2017).

111. Kobata, A. The N-linked sugar chains of human immunoglobulin G: their unique pattern, and their functional roles. *Biochim. Biophys. Acta* **1780**, 472–478 (2008).

112. Jefferis, R. Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog.* **21**, 11–16 (2005).

113. Tetala, K. K., and van Beek, T. A. Bioaffinity chromatography on monolithic supports. *J. Sep. Sci.* **33**, 422–438 (2010).

114. Burnouf, T., Goubran, H. & Radosevich, M. Application of bioaffinity technology in therapeutic extracorporeal plasmapheresis and large-scale fractionation of human plasma. *J Chromatogr B Biomed Sci Appl* **715**, 65–80 (1998).

115. Gupalova, T. V., Lojkina, O. V., Palagnuk, V. G., Totolian, A. A. & Tennikova, T. B. Quantitative investigation of the affinity properties of different recombinant forms of

protein G by means of high-performance monolithic chromatography. *J Chromatogr A* **949**, 185–193 (2002).

116. van de Bovenkamp, F. S., Hafkenscheid, L., Rispens, T. & Rombouts, Y. The Emerging Importance of IgG Fab Glycosylation in Immunity. *The Journal of Immunology* **196**, 1435–1441 (2016).

117. Bondt, A. *et al.* Immunoglobulin G (IgG) Fab glycosylation analysis using a new mass spectrometric high-throughput profiling method reveals pregnancy-associated changes. *Molecular & Cellular Proteomics* **13**, 3029–3039 (2014).

118. Keser, T., Pavic, T., Lauc, G. & Gornik, O. Comparison of 2-aminobenzamide, procainamide and RapiFluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis. *Front Chem* **6**, 324 (2018).

119. Ruhaak, L. R., Steenvoorden, E., Koeleman, C. A. M., Deelder, A. M. & Wuhrer, M. 2-Picoline-borane: A non-toxic reducing agent for oligosaccharide labeling by reductive amination. *Proteomics* **10**, 2330–2336 (2010).

120. Afiuni-Zadeh, S., Rogers, J. C., Snovida, S. I., Bomgarden, R. D. & Griffin, T. J. AminoxyTMT: A novel multi-functional reagent for characterization of protein carbonylation. *Biotechniques* **60**, 186–196 (2016).

121. Reiding, K. R., Blank, D., Kuijper, D. M., Deelder, A. M. & Wuhrer, M. High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification. *Anal Chem* **86**, 5784–5793 (2014).

122. Sekiya, S., Wada, Y. & Tanaka, K. Derivatization for stabilizing sialic acids in MALDI-MS. *Anal Chem* **77**, 4962–4968 (2005).

123. Wysocki, V. H., Tsaprailis, G., Smith, L. L. & Breci, L. A. Mobile and localized protons: A framework for understanding peptide dissociation. *Journal of Mass Spectrometry* **35**, 1399–1406 (2000).

124. Alley Jr, W. R. & Novotny, M. V. Glycomic analysis of sialic acid linkages in glycans derived from blood serum glycoproteins. *J Proteome Res* **9**, 3062–3072 (2010).

125. Huffman, J. E. *et al.* Comparative performance of four methods for high-throughput glycosylation analysis of immunoglobulin G in genetic and epidemiological research. *Molecular and Cellular Proteomics* **13**, 1598–1610 (2014).

126. Kattla, J. J. *et al.* Protein Glycosylation (2nd Edition). in *Comprehensive Biotechnology* (ed. Murray Moo-Young) vol. 3 467–486 (Elsevier, 2011).

127. Ruhaak, L. R. *et al.* Glycan labeling strategies and their use in identification and quantification. *Anal Bioanal Chem* **397**, 3457–3481 (2010).

128. Han, L. & Costello, C. E. Mass spectrometry of glycans. *Biochemistry (Moscow)* **78**, 710–720 (2013).

129. Pabst, M. & Altmann, F. Glycan analysis by modern instrumental methods. *Proteomics* **11**, 631–643 (2011).