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Glycosylation signatures of type 2 diabetes complications and metabolic syndrome

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CHAPTER

6

Discussion and Perspectives

Glycomics has emerged as a promising field for identifying advanced biomarkers for analyzing diabetes mellitus and other metabolic diseases in clinical and medical research. We have shown that analyzing changes in the profiles of complex sugars (*N*-glycans in this thesis) can reveal associations that may be useful for understanding type 2 diabetes (T2D) and other metabolic illnesses (**Chapters 2,5, ¹**). These association findings suggest that *N*-glycan profiling has biomarker potential and may facilitate earlier diagnosis and more effective treatments in the future. The pathophysiological aspects of T2D and its vascular complications are not fully understood because it is a complex multifactorial long-term metabolic condition ^{2,3}. Therefore, glycomics research may improve our understanding of diabetes and its management.

The primary objective of this thesis was to obtain the association of *N*-glycans with T2D vascular complications and to investigate the connection between *N*-glycan biomarkers and metabolic syndrome (MetS). The findings were based on the analysis of *N*-glycans released from plasma proteins to assess their associations with MetS and T2D complications (**Chapter 2 and 3**). An important point differentiating the experimental chapters of this thesis is that **Chapters 2 and 3** are based on methods published in previous studies ^{1,4}, whereas **Chapters 4 and 5** include method development and tailoring aspects. A similar analytical approach used in **Chapter 3** was extended to analyze released *N*-glycans from immunoglobulin G (IgG) (**Chapter 4**). For the analysis of released glycans from dried blood spots (DBS), a similar analytical approach with some modifications was employed (**Chapter 5**).

Thus, the chapters of this thesis cover different scopes, as some of them (**Chapters 2 and 3**) primarily address a biological question, while for others (**Chapters 4 and 5**) there is also an intermediate goal of demonstrating the validity of the method due to new methods which we developed. **Chapter 5**, in particular, is primarily dedicated to development and showcasing of the applicability of a method.

In the following the research of this thesis will be put into perspective, and considerations will be made regarding the direction future research may take, and an assessment will be conducted on the potential for translating results into clinics for T2D patients.

6.1 MetS risk factors

The increasing prevalence of T2D and MetS arises from a sedentary lifestyle, poor diet, obesity, and aging. Sleep issues, chronic stress, and certain medications also contribute ^{5–10}. It is worth noting that certain risk factors, such as aging, are non-modifiable, while others can be addressed through lifestyle changes, such as regular exercise and adherence to a healthy diet. Hence, a multifaceted approach is necessary to manage and prevent T2D and MetS, encompassing interventions targeted at addressing the aforementioned factors to mitigate the growing burden of these conditions on public health. *N*-glycans play a role in various biological processes, such as receptor oligomerization, ligand binding and signaling, due to their complex and diverse functions

^{11,12}. Taking a closer look at this complexity, our exploration shows that the *N*-glycosylation of plasma proteins has been linked to MetS and diabetes, both with and without complications (**Chapters 2-4**, ¹³). Concerning the specified requirement, **Chapter 2** of our study aimed to investigate and characterize the plasma *N*-glycome among both healthy individuals and those with MetS and suboptimal health status (SHS) within the Ghanaian population consisting of 262 individuals. This is not the first study to analyze *N*-glycome profiling related to metabolic health, but it is exceptional in its focus on populations isolated by geography, climate, and genetics, particularly those in West Africa.

Chapter 2 revealed an association between *N*-glycans, SHS and MetS, wherein MetS shows elevated levels of more complex *N*-glycan structures on plasma glycoproteins. Additionally, there is an observed rise in the plasma concentration of glycoproteins that mostly carry these glycans. The study revealed that an increase in sialylation and galactosylation of *N*-glycan structures was linked to higher BMI and obesity, which are important parameters for characterizing MetS, SHS and T2D ^{5,14,15}. These associations align with similar findings from other populations, such as Scottish, Chinese, and Croatians, where trigalactosylated, trisialylated, and triantennary glycans were positively associated with BMI.

Moreover, modest differences in *N*-glycan levels between low and high SHS groups were observed. Notably, the tetrasialylated glycans were more abundant in high SHS. However, when considering the derived traits of *N*-glycans and accounting for multiple testing, no significant differences were observed. It is worth noting that participants diagnosed with MetS displayed higher total SHS scores in comparison to those without MetS, which adds an interesting dimension to the observed associations.

In addition to the aforementioned findings, an increase in antennary fucosylated and triantennary structures in MetS patients was observed, which may indicate the presence of inflammation.

Identifying and controlling specific *N*-glycosylation patterns may lead to the development of new biomarkers for MetS and related disorders. Moreover, given the role of *N*-glycosylation in protein function and structure, interventions addressing *N*-glycosylation, including carbohydrate food supplements, could offer novel therapeutic approaches for these conditions. One notable example is the use of glycan supplementation therapeutics, which have shown promising results in reducing disease activity in carbamoyl-phosphate synthetase 2 aspartate transcarbamylase and dihydroorotase (CAD)- congenital disorders of glycosylation (CDG) disease. Additionally, a separate study revealed that by supplementing food with GlcNAc, it may be possible to reprogram the glycosylation patterns in kidney tissue, leading to the control and prevention of autoimmune pathogenesis ¹⁶⁻¹⁹. However, a deeper understanding of the underlying mechanisms linking total plasma *N*-glycosylation and MetS is required before any clinical application can be pursued.

N-glycosylation findings come with significant and broad implications. As it was mentioned, our study (**Chapter 2**) focused on samples from populations in West Africa that are isolated, and compared these findings with results from diverse populations. Interestingly, we observed some variations in the extent of correlation or levels of *N*-glycans among different populations. In **Chapter 2**, we controlled for confounding factors, including age, sex, and BMI, in line with previous studies highlighting their impact on *N*-glycome²⁰.

Our decision to focus on specific confounding variables; age, sex, and BMI; was guided by the robust evidence indicating their influence on *N*-glycan profiles^{20–24}. Although our findings in **Chapter 2** suggested potential effects of additional factors such as education level, we chose to prioritize investigating confounding parameters with well-established effects demonstrated across numerous studies. There are limitations to our study owing to the small sample size and the absence of control for heritability relationships. Therefore, there is a necessity for a replication study that includes geographically isolated populations as well as samples from diverse ethnicities and races such as Asia and Europe, in order to increase the number of participants and thoroughly investigate the impact of genetic control and heritability relationships.

While our article in **Chapter 2** explored the relationship between *N*-glycosylation and MetS, as well as the effect of SHS, and despite our efforts to mitigate experimental errors and bias by randomizing samples on multiple plates, the use of a cross-sectional design restricts our ability to determine whether aberrant *N*-glycan profiles are the cause or consequence of metabolic abnormalities. Thus, longitudinal studies are needed to overcome these limitations and yield more reliable results. In essence, our findings necessitate another replication project to address these constraints, thereby providing sufficient power to make our assumptions definitive and applicable across all populations.

6.2 *N*-glycosylation in T2D complications

It is known that TPNG traits may serve as a useful biomarker for T2D risk, as some studies have suggested^{13,25}.

Despite preventive attempts, T2D continues to carry a notable risk of macrovascular and microvascular complications, placing significant challenges on affected individuals. The role of TPNG in these complications remains relatively unknown, making its relationship with T2D complications a specific area of interest. **Chapter 3** of this thesis demonstrates similarities between incident and prevalent complications. A high level of bisection on a group of diantennary (A2FS0B) glycans were shown to have the highest correlation with the prevalence of cardiovascular disease. In the context of prevalent nephropathy, increased 2,6-sialylation on triantennary glycans was especially notable. Regarding retinopathy, the most significant and only

glycans that had a meaningful negative association with retinopathy was the the number of mannoses within the hybrid glycans (MHy).

Our study pioneers the investigation into the correlation between TPNG and various macrovascular and microvascular complications in T2D. Articles published after our work also highlight the significance of this topic. For example, one study discusses the crucial role of glycosylation in cardiovascular diseases, specifically atherosclerosis and myocardial infarction ²⁶. It emphasizes the significance of glycan structures in disease progression and suggests targeting glycosylation processes as a potential therapeutic strategy. Chatham et al. also mentioned that protein glycosylation is crucial for regulating various homeostatic functions in the cardiovascular system ²⁷. Moreover, Ren et al. highlighted the importance of *N*-glycosylation in kidney diseases, particularly in renal structure and function, with mass spectrometry-based techniques playing a crucial role in analyzing *N*-glycosylation for early diagnosis and treatment ²⁸. Extending across two extensive, independent cohorts with follow-ups, our research (**Chapter3**) also illuminates a potential limitation in not exploring the influence of medication use on complications. In a separate study, we established associations between TPNG and the use of drugs, suggesting a role of medication in the current findings. Furthermore, the limited number of incident cases for each complication, particularly in CVD and retinopathy, represents another constraint in our study. Balanced studies with larger sample sizes are required for conducting more intricate investigations and revealing the influence of medication use.

While employing an automated high-resolution method and successfully implementing sialic acid derivatization for quantifying and distinguishing sialic acid linkages, our technique could not tell the difference between other isomeric structures, such as galactose linkage isomers. This highlights the need for further refinement of the analytical methodology.

Although total *N*-glycomic changes provide a wealth of information on the association between *N*-glycans and various diseases, they do not disclose the specific proteins involved and the findings can be affected by differences in the relative abundance of proteins in the bloodstream. It is also important to note that the glycosylation of certain plasma proteins, such as IgG, may have implications for the pathophysiology of T2D ^{29,30}. On the other hand, T2D is recognized as a pro-inflammatory disease and low-level inflammation is a prevalent characteristic of T2D ³¹. The persistent and mild inflammation observed in T2D may play a role in the onset of other inflammatory disorders and ailments, including cardiovascular diseases ³².

Chapter 4 of this thesis presents evidence that IgG *N*-glycosylation is associated with an increased prevalence and future development of macro- and microvascular complications of diabetes. Our investigation revealed negative associations between galactosylation and prevalent as well as incident nephropathy and macrovascular disease. Moreover, galactosylation was also negatively linked with both prevalent and incident retinopathy. The reduction in IgG galactosylation may indicate a common feature of pro-inflammatory disease, as reported in several studies ^{33–35}. Similarly, lower galactosylation has been demonstrated for individuals with T2D compared to

those without the condition ²⁹. In addition, our results indicated inverse correlations between sialylation and incident nephropathy, as well as between sialylation and prevalent macrovascular complications. Consistent with our findings, a decrease in sialylation was also documented in various other conditions, including inflammatory bowel disease (IBD), chronic kidney disease (CKD), colorectal cancer, next to T2D ^{29,34–36}. Incorporation of sialic acid into IgG results in a functional switch from a pro-inflammatory to an anti-inflammatory state, although a molecular, mechanistic understanding of this switch is still elusive ³⁷. Of note, the deficiency of galactose translates into lower levels of sialic acid, as sialylation is predominantly reliant on terminal galactose residues as acceptor sites ^{38,39}.

Interestingly, other researchers ^{40–42} demonstrated glycan associations similar to those described in this thesis. For example, Hoshi et al. explored the association between IgG *N*-glycans and the risk of future CVD. They identified specific IgG glycan traits that were either positively or inversely associated with CVD risk, such as digalactosylation and monosialylation traits being inversely associated with CVD risk ⁴⁰. Additional work by Wang et al. reported similar patterns of IgG galactosylation and sialylation in T2D and hypertension, suggesting a bidirectional causal association between T2D and hypertension mediated by IgG *N*-glycosylation ⁴¹.

While our study (**Chapter 4**) has the strength of using three large independent populations with a prospective nature allowing us to explore the chronological connection between IgG *N*-glycan patterns and diabetic complications, there are several limitations that deserve consideration. IgG *N*-glycosylation was measured only at baseline, potentially missing changes that could occur during follow-up. Additionally, the prospective analyses note the possibility of changes in influencing factors during follow-up and lack serum markers of inflammation, hindering a more in-depth investigation. The study was also underpowered for macrovascular complications due to their rarity, and the presence of multiple vascular complications at baseline raised concerns about potential confounding associations. To address these limitations, future research should involve multiple measurements during follow-up, integrate serum markers of inflammation, and consider larger sample sizes or multi-center collaborations to boost statistical power. Adjusting for the presence of multiple complications in analyses would enhance result accuracy, offering a more robust understanding of the relationship between IgG *N*-glycans and diabetic vascular complications.

6.3 *N*-glycan profiles as potential biomarker for T2D and MetS

It is intriguing to explore the similarities and differences in *N*-glycomic patterns between T2D and MetS. Pongracz et al., in a study released after ours, reexplored blood *N*-glycomic signatures from the past decade, integrating findings across major diseases and discussing challenges and translational examples. They state that while inflammation is a common feature in various

diseases, including T2D, cancers, autoimmune disorders, and other metabolic conditions, these diseases come with diverse alterations in glycosylation patterns ⁴³.

When we compared the results from one of our other studies on Ghanaian individuals' plasma *N*-glycans in T2D patients and healthy individuals with the results from MetS (**Chapter 2, 1**), it was revealed that the decrease in diantennary galactosylation, and diantennary *N*-glycans, as well as the increase in high branching, tri-galactosylated, antennary fucosylated, and triantennary *N*-glycans, showed a similar trend in both MetS and T2D samples compared to the control group. This finding is expected, as T2D is associated with MetS, and suggests that there may be shared underlying mechanisms and pathways driving the changes in *N*-glycomic patterns in both conditions.

However, a few additional derived traits were also significantly different in T2D individuals compared to those with MetS. Notably, monosialylated diantennary *N*-glycans, which is a marker of individuals with MetS, did not exhibit a difference in T2D subjects compared to the control group.

By comparing the *N*-glycan profiles of individuals with T2D and MetS to those of healthy individuals, there lies the prospect of a better diagnostic test that could be not only more accurate but also more reliable for identifying these conditions. This could potentially lead to earlier detection and more effective treatment of T2D (with and without complications) and MetS.

However, it is important to consider the factors that contribute to changes in *N*-glycomic profiles. The altered *N*-glycan structures observed in T2D (with and without complications) and MetS may be attributed to various factors, including inflammation, hypertension, and dyslipidemia ^{44,45}. For example, previous studies have shown that inflammation, as reflected in increased C-reactive protein (CRP) levels, is associated with changes in *N*-glycan structures ⁴⁶. In addition, environmental and lifestyle factors such as diet and physical activity may influence *N*-glycomic profiles ^{21,22} and affect development of T2D and MetS ⁷. Therefore, more research is needed to fully understand the underlying mechanisms driving these changes.

6.4 DBS glycomics for clinical translation

The preceding chapters have emphasized the importance of timely and accurate detection of T2D and its complications, as well as the need for reliable and robust diagnostic methods to enable effective management and prevention of long-term health consequences. Complications from T2D may be eliminated or at least delayed with early detection and treatment of T2D ^{47–49}.

While blood and urine tests are currently widely employed in clinical assessments, DBS tests hold promise as a potentially useful screening tool for patients. The ease of collecting and transporting DBS samples allows for their use in disease screening and monitoring, especially in settings with limited resources.

DBS analysis holds great potential in detecting a diverse array of diseases. Different studies have demonstrated the ability of DBS analysis to detect a range of diseases, including diabetes, infectious diseases (such as HIV), inherited metabolic disorders (such as phenylketonuria), genetic disorders (such as sickle cell anemia), and hormonal disorders (such as hypothyroidism) ^{50–54}.

In general, the potential of DBS analysis to detect different diseases makes it a promising tool for disease screening and monitoring in both developed and developing countries ⁵⁵. Furthermore, some studies have analyzed the *N*-glycan profile of DBS from healthy individuals and demonstrated that the *N*-glycan profile from DBS and plasma samples (which are obtained from venous blood collection) are similar ^{56–58}.

Additionally, the IgG *N*-glycan profile of DBSs demonstrates stability under different storage conditions, indicating that DBS samples serve as a reliable and stable source material for robust IgG *N*-glycan analysis ^{56,58}. The association of TPNG and IgG released *N*-glycans with T2D and corresponding complications were previously validated for plasma and serum samples (**Chapter 3,4**, ^{13,29}). Thus, DBS glycomic analysis has the potential to be used as a promising method for detecting and monitoring T2D (with and without complications) biomarkers.

Commercially available diabetes test kits (or more commonly, home blood sugar tester kit for diabetics), are capable of measuring blood glucose levels as molar concentration, mmol/L, or mass concentration, mg/dL, with reasonably accurate results, depending on the vendor ^{59–61}. It is possible to utilize a similar process for home-based collection of DBS samples, provided that a suitable kit is used. After collecting DBS samples from each individual, they can be transported to a laboratory where they will be analyzed to provide results within a maximum of 14 days. While the convenience of obtaining instant at-home glucose level readings may first seem advantageous when compared to the more time-consuming procedure of submitting samples for DBS analysis and waiting for 14 days, it is important to consider that DBS *N*-glycans may provide a more comprehensive and reliable profile. Prediabetes, which is frequently unnoticed, can be accurately identified by DBS. Furthermore, DBS analysis could provide valuable insights into issues associated with diabetic complications that remain undetectable through the home testing approach. As a next step towards clinical translation, analysis of a great number of DBS samples is needed, ensuring that the framework aligns seamlessly with the specific requirements and challenges of the healthcare environment. Thus, it should be noted that further work is still required for the practical solution to be fully implemented and functional in a clinical setting.

Based on the aforementioned, the TPNG/IgG released *N*-glycans profile obtained from DBSs can provide beneficial information about (pre-)diabetes level, T2D complications and even MetS. This idea can be partially supported by the findings presented in **Chapter 5** of this thesis, which

provide probable evidence that certain *N*-glycan structures found in dried blood samples could be associated with pre-diabetes and diabetes.

In **Chapter 5**, our primary focus was on assessing the stability of *N*-glycans in DBS samples across diverse preparation methods. Regardless of the DBS preparation approach; whether fresh blood, whole frozen blood, or separated frozen-blood cells with corresponding frozen plasma; *N*-glycosylation profiles and relative abundances stayed unaffected. Moreover it is worth mentioning that by incorporating plasma standard samples into our analysis, we were able to verify the accuracy and reliability of our measurements, thus affirming the validity of our findings. We conducted the same approach on samples from pre-diabetic and diabetic individuals that were stored under specific conditions, with the purpose of demonstrating that our approach can be applied to real pre-diabetic and diabetic samples. Our findings suggested the potential of DBS *N*-glycans to distinguish between pre-diabetic and diabetic individuals. Notably, we used DBS from a combination of separated frozen-blood cells and corresponding frozen plasma obtained from pre-diabetic and diabetic samples that had been stored for an extended period. This showcases adaptability of our approach in challenging scenarios where collecting fresh blood for DBS is impractical or where the primary blood samples were not originally obtained for experiments involving DBS analysis.

It's essential to clarify that our study did not aim to assess biological differences between pre-diabetic and diabetic samples due to the limited number of samples in each group. Instead, similar to previous publications from our group, such as the work by Gudelj et al. and Vreeker et al., which explored the use of DBSs for estimating biological age and assessing the stability of the *N*-glycome from DBS under different storage conditions, respectively^{56,57}, our main objective was to demonstrate the robustness of the method with varied storage conditions. Additionally, we aimed to showcase the potential of DBS for analyzing diabetic or pre-diabetic samples. The results we presented from this comparison (between pre-diabetic and diabetic individuals) are very preliminary. Certainly, to confirm these results, further experiments with a larger sample size are needed to compare the biological characteristics of pre-diabetic and diabetic individuals.

In our current research, we observed trend towards increased fucosylation, bisection, and galactosylation in samples from persons with diabetes compared to pre-diabetic ones. Conversely, we noted a trend towards lower sialylation in diabetic samples compared to pre-diabetic samples. Notably, certain findings, such as the rising trend of galactosylation in diabetic samples, align with previous research indicating an elevated risk of T2D associated with increased plasma galactosylation⁶². However, as previously mentioned, the uneven sample distribution and relatively small sample size in our study could explain contradictory results on sialylation and lack of significant associations, and may indicate that the results of this comparison might not be reliable. These limitations suggest a need for further investigation with a larger cohort to replicate our approach and validate the potential of using DBS for analysing pre- and diabetic

samples. While demographic factors are important considerations in epidemiological studies, our focus was primarily on the glycosylation patterns themselves and exploring the differences in *N*-glycome profiles between different blood preparation approaches and its applications on pre- and diabetic samples rather than the influence of demographic variables. Moreover, given the complexity of *N*-glycome analysis and the limited sample size in our study, adjusting for confounding variables could introduce additional complexity and potential bias into our analysis. Our study may not have the statistical power necessary to adequately adjust for all potential confounding factors. In future larger-scale research, there is a need to explore the influence of confounders (like age, sex, etc.) on *N*-glycome variations.

When implementing a DBS *N*-glycomics strategy, in addition to measuring glucose, insulin, and glycated hemoglobin (HbA1c), physicians may gain a better understanding of a patient's diabetes stage, and more information about potential diabetic complications for the corresponding individual. Improved monitoring of T2D patients ultimately leads to better patient outcomes ⁶³. Personalized medicine approaches could utilize the DBS *N*-glycan profile to tailor treatment strategies to individual patients based on their unique biomarker profiles. Finally, DBS *N*-glycan analysis could potentially be applied in screening efforts of novel therapeutic targets for T2D and its complications.

To make commercial use of DBS, similar to GlycanAge products (a commercial non-clinical use of *N*-glycan profile to determine an individual's biological age) ^{33,64}, there is a need to evaluate results based on a unique index composed of specific *N*-glycan structure variables ³³, while adhering to relevant in vitro diagnostics regulations. These specific *N*-glycans should be the *N*-glycan structures that exhibit the most significant association with T2D and its complications. However, due to its intended clinical application, careful consideration should be given to the selection of these indexes. Regulatory criteria govern diagnostic test development, validation, approval, and commercialization. These regulations ensure that diagnostic tests are both accurate and of high quality ^{65,66}.

Researchers are actively searching for new glycomics biomarkers. The goal is to address the need for diagnostic tests that can detect protein-specific glycosylation alterations of major plasma glycoproteins at an early stage. They aim to analyze the structure of glycan(s), and collect data from individuals at various timepoints in a longitudinal setting. By doing so, researchers hope to provide valuable insights with potential for clinical translation (**Chapter 4**, ^{67,68}). The use of test evaluation frameworks is strongly encouraged. The development of diagnostic tests and their subsequent approval are governed by health authorities, such as, the Food and Drug Administration (FDA) in the United States, or the European Federation of Laboratory Medicine (EFLM) and European Medicines Agency (EMA) in the European Union.

In the context of T2D diagnostics, a comprehensive glycomics assessment and an evaluation of potential metabolic factors are necessary ^{65,66}. Moreover, additional approaches can be implemented to enhance the availability and applicability of DBS for both patients and healthcare professionals. For instance, *N*-glycan antennary fucosylation has been identified as a clinical biomarker for hepatocyte nuclear factor-1 alpha (HNF1A)-maturity-onset diabetes of the young (MODY) ^{69,70}. Consequently, constructing a library of *N*-glycan profiles from healthy individuals, those with T2D, and those with HNF1A-MODY would serve to enhance the diagnostic capacity of *N*-glycans released from DBSs, thereby facilitating improved patient stratification and treatment recommendations.

Therefore, *N*-glycomic profiling via the DBS approach has great potential to be implemented as a valuable tool for early detection and monitoring of T2D and its complications in the clinical and commercial setting.

Although more research and development are required to potentially translate our findings into a clinical setting, we have here demonstrated that *N*-glycans from total plasma proteins or IgG are promising biomarkers in the diagnostics of MetS, T2D and T2D complications. Additionally, we made progress toward introducing the *N*-glycan profiling DBS approach in context of T2D. In conclusion, our findings pave the way for the development of *N*-glycan based diagnostic tools that enable an early detection and monitoring of MetS, T2D and its complications.

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