2018

N-Glycosylation profiles as a risk stratification biomarker for Type II Diabetes Mellitus and its associated factors

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N-GLYCOSYLATION PROFILES AS A RISK STRATIFICATION BIOMARKER FOR TYPE II DIABETES MELLITUS AND ITS ASSOCIATED FACTORS

Submitted for the Degree of

Doctor of Philosophy

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BSc (Hons), MSc

Edith Cowan University

School of Medical and Health Sciences

2018

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DECLARATION

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ABSTRACT

Worldwide, the prevalence of cardiometabolic diseases, particularly type II diabetes mellitus (T2DM), and to a lesser extent, metabolic syndrome (MetS), has increased dramatically. Despite this increase, there is still a lack of robust biomarkers for cardiometabolic diseases to secure better clinical outcomes. The enzymatic attachment of oligosaccharides (glycans) to proteins-glycosylation is of metabolic and physiological significance, as exploring aberrations of glycosylation profiles can reveal novel biomarkers. In parallel, this process could also explain the biological mechanisms that underpin a suboptimal health status (SHS), a reversible subclinical stage of a cardiometabolic disease. However, studies on the correlation between glycosylation and MetS/T2DM are scarce and none has thus far been performed on a West African population. Thus, the overall aim of this thesis was to explore complementary biomarker panels of healthy and diseased patients considered relevant to Ghanaian residents. The thesis is structured in the form of five related studies, each addressing a specific aim. From January 2016 to October 2016, a longitudinal case-control study comprising 253 T2DM patients and 260 controls, aged 18-80 years was conducted in Ghana. Fasting plasma samples were collected for clinical assessment, after which plasma N-glycans were analysed by Ultra-Performance Liquid Chromatography (UPLC) and statistical analyses performed. Central adiposity, underweight, high systolic blood pressure (SBP), high diastolic blood pressure (DBP) and high triglycerides (TG) were found to be independent risk factors associated with high SHS after adjusting for age and gender **(Study I)**. SHS score was associated with age, physical inactivity, fasting plasma glucose (FPG), TG and MetS. MetS was associated with increased high branching (HB), trigalactosylated (G3), antennary fucosylated (FUC_A), triantennary (TRIA) and decreased low branching (LB) glycan structures **(Study II)**. The levels of HB, G3, FUC_A, and TRIA N-glycans were increased in T2DM whereas levels of LB, non-sialylated (S0), monogalactosylation (G1), core fucosylation (FUC_C), biantennary galactosylation (A2G) and biantennary (BA) N-glycans were decreased compared to controls **(Study III)**. Biguanides alone, or in combination with sulfonylurea and thiazolidinedione, did not improve glycaemic status at follow-up. Many participants using angiotensin converting enzyme inhibitors achieved desired targets for blood pressure control while statins were effective for control of plasma lipids **(Study IV)**. At a population level, the variability of N-glycan structures ranged from 11% to 56% at both baseline and follow-up, with an average coefficient of variation of

28% and 29%, respectively. The intra-individual N-glycan peak (GP) variations were minor except for GP1 and GP29. However, there were no statistically significant differences in N-glycosylation profiles from baseline to follow-up **(Study V)**. This thesis shows an association between SHS and MetS/T2DM while MetS and T2DM are characterised by increased levels of complex N-glycan structures, and these structures are stable in T2DM over six months. Many of the findings in this thesis agree with earlier studies from Chinese and Croatian populations with major differences attributed to genetic and environmental factors. Future longitudinal studies are required to provide a better understanding of the transition from SHS to T2DM, as well as to validate N-glycans as generic risk stratification biomarkers for a general population.

Acknowledgements

Firstly, I will like to express my sincere appreciation to my principal supervisor, Professor Wei Wang, and co-supervisor, Associate Professor Peter Roberts, for their immense and continuous support throughout the PhD journey. Their advice, comments and encouragement were instrumental to completing this thesis. To my colleague, Alyce Russell, I say thank you for the immeasurable support you provided me over the past years. I thank my teammates: Enoch Odame Anto, Xingang Li, Hao Wang, Siqi Ge, and Xueqing Wang for their discussions and contributions to my work. To all other colleagues in the PhD suite of building 21, I say thank you. I wish to thank our collaborators at Genos Glycoscience Research Laboratory, Zagreb, Croatia for performing the N-glycan analysis.

My sincere appreciation also goes to the staff of the Diabetes Clinic of the Komfo Anokye Teaching Hospital (KATH). In particular, I will like to thank Dr Osei Sarfo Kantanka, Dr Fred Stephen Sarfo, Mrs Agnes, Shadrach, Gideon, amongst others, for their immense support during the data collection phase of this work. I sincerely thank Dr Albert Dompreh for his advice and giving me space to store my samples. My profound gratitude goes to the participants, both T2DM sufferers and healthy controls, for not only giving their medical and demographic information, but also providing blood samples for analyses.

Special appreciation also goes to Dr Samuel Asamoah Sakyi, Associate Professor Francis Agyemang Yeboah, Dr William KBA Owiredu, Dr Edwin Ferguson Laing and the entire staff at the Department of Molecular Medicine of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

My heartfelt thanks goes to Dr Kwasi Frimpong for his encouragement, advice and support throughout my stay in Australia. In addition, I thank Dr Francis Atta Kuranchie and Isabella Kuranchie, Dr Daniel Doe, Dr Esther Adama and Dr Emmanuel Aboagye for their encouragements. To Mr. Kwaafo Awuah Mensah, Mr Wilfred Sefah, Mr. Isaac Kosi, Mr. Hayford Ofori, Mrs Mary Afi Mensah and Mr. Yakubu Sumaila, I say thank you for your motivation, encouragement and company. Further, I thank Mrs Joanne Hoffsten for being a mother and given me overwhelming care, support and advice.

To Mr. Joseph Sim of the Graduate Research School (GRS), I say thank you for your timely advice and your willingness to talk to me anytime I approached you. Finally, I thank Dr Amanda Harris, communication adviser (GRS), for proofreading parts of my manuscripts and helping me to improve on my writing skills.

Source of Funding

This project was supported partially by the Joint Project of the Australian National Health & Medical Research Council and the National Natural Science Foundation of China (NHMRC APP1112767-NSFC 81561128020), National Natural Science Foundation of China (81771207, 81370083, 81673247, 81573215), Edith Cowan University Collaboration Enhancement Scheme 2017 (Round 1), the National Key Technology Support Program of China (2012BAI37B03); as well as by funding from the European Structural and Investments funds for project "Croatian National Centre of Research Excellence in Personalized Healthcare" (contract No. KK.01.1.1.01.0010) and funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant for project GlySign (contract No. 722095).

List of Publications

Research articles

I. **Adua E**, Memarian E, Russell A, Trbojević-Akmačić I, Gudelj I, Jurić J, Roberts P, Lauc G, and Wang W (2018). High throughput profiling of whole plasma N-glycans in type II diabetes mellitus patients and healthy individuals: A perspective from a Ghanaian population. *Archives of biochemistry and biophysics Arch Biochem Biophys.* 661:10-21.

II. **Adua E**, Anto E. O, Roberts P, Kantanka, O. S, Aboagye E, and Wang W. (2018). The potential of N-glycosylation profiles as biomarkers for monitoring the progression of Type II diabetes mellitus towards diabetic kidney disease. *Journal of Diabetes & Metabolic Disorders*, 365:1-14.

III. **Adua E**, Roberts P and Wang W (2017). Incorporation of suboptimal health status as a potential risk assessment for type II diabetes mellitus: a case-control study in a Ghanaian population. *EPMA Journal*, *8* (4), 345-355.

IV. **Adua E**, Roberts P, Sakyi SA, Yeboah FA, Dompreh A, Frimpong K, Anto EO, and Wang W (2017). Profiling of cardio-metabolic risk factors and medication utilisation among Type II diabetes patients in Ghana: a prospective cohort study. *Clinical and Translational Medicine, 6* (1), 1-12.

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2. **Adua E**, Russell A, Roberts P, Wang Y, Song M and Wang W (2017). Innovation analysis on postgenomic biomarkers: glycomics for chronic diseases. *OMICS: A Journal of Integrative Biology*. *21* (4), 183-196.

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Science/AAAS Washington DC, 36–39. doi.org/10.1126/science.354.6319.1601-b (*Contributed equally).

3. Russell A, **Adua, E**, Ugrina I, Laws S, Wang W. (2018). Unravelling immunoglobulin G Fc N-glycosylation: A dynamic marker potentiating predictive, preventive and personalised Medicine. *International Journal of Molecular Sciences*. *19*(2), 1-19.

Manuscripts under review

1. **Adua E**, Memarian E, Russell A, Trbojevic-Akmacic A, Gudeji I, Juric J, Roberts P, Lauc G. and Wang W. Utilisation of N-glycosylation profiles as risk stratification biomarkers for suboptimal health status and metabolic syndrome.

2. **Adua E**, Memarian E, Russell A, Trbojevic-Akmacic I, Gudeji I, Juric J, Roberts P, Lauc G. and Wang W. N-Glycosylation Profiling of Type II Diabetes Mellitus from Baseline to Follow-up: An Observational Study in a Ghanaian Population.

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DEDICATION

This thesis is dedicated to my sisters Juliana, Lydia, Linda, Vida, Alice and my brother Frederick.

List of Abbreviations

2-AA	2-aminobenzoic acid
2-AB	2-aminobenzamide
2-AP	2-aminpyridine
ACEI	Angiotensin converting enzyme inhibitors;
ADA	American diabetes association
ANDSA	7-amino-1, 3-naphthalene disulfonic acid
ANOVA	Analysis of variance
ANS	2-aminonapthalene-1-sulfonic acid
ANTS	2-aminonaphthalene trisulfonic acid
APTS	1-aminopyrene-3, 6, 8-trisulfonic acid
ARB	Angiotensin II receptor blockers
Asn	Asparagine
AUC	Area under the curve
BH	Benjamini-Hochberg
BIG	Biguanides
BMI	Body mass index
CAD	Central acting drugs
CCA	Canonical correlation analysis
CCB	Calcium channel blockers
CCSD	Complex carbohydrate structure database
CDG	Congenital disorders of glycosylation
CDKAL 1	Cyclin dependent kinase A like 1
CE	Capillary electrophoresis
CFG	Consortium for functional glycomics
COPD	Chronic obstructive pulmonary disease
CR	Coronary risk
CRP	C-reactive protein
CZE	Capillary zone electrophoresis
DAP	2, 6-diaminopyridine
DBP	Diastolic blood pressure
DMSO	Dimethylsulfoxide
Dol-PP	Dolichol phosphate
DSA-FACE	DNA sequencer-aided flourophore-assisted carbohydrate electrophophoresis
DTT	Dithiothreitol
ECM	Extracellular matrix
Endo	N-endoglycosidase
EOC	Epithelial ovarian cancer
ER	Endoplasmic reticulum
ESI	Electrospray ionization
ESI-MS/MS	Electrospray ionization tandem mass spectrometer
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FPG	Fasting plasma glucose
FT-ICR-MS	Fourier transform ion cyclotron
FUC	Fucose
FUT 8	Fucosyltransferase 8
G1cNAc	N-acetylglucosamine
GAG	Glycosaminoglycan
GCC	Graphitized carbon chromatography
GlcNAc	2 N-acetylglucosamine residues
Glu	Glucose
Gly	Glycine
GnT V	*N*-acetylglucosaminyltransferase V
GPC	Gel permeation chromatography
GU	Glucose unit
GWAS	Genome-wide association study

HbA1c	Glycated haemoglobin
HBP	Hexosamine biosynthetic pathway
HDL-c	High-density lipoprotein cholesterol
HILIC	Hydrophilic interaction liquid chromatography
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
ICD	International Classification of disease
IDF	International Diabetes Federation
IRS	Insulin receptor substrate
IQR	Interquartile range
KATH	Komfo Anokye Teaching Hospital
LC	Liquid chromatography
LDL	Low-density lipoprotein
LIF	Laser induced florescence
MALDI	Matrix assisted laser desorption ionization
MALDI-TOF	Matrix assisted desorption ionization-time of flight
MEKC	Micellar electrokinetic chromatography
MetS	Metabolic syndrome
MS	Mass spectrometry
MS-MSI	Mass spectrometry-mass spectrometry imaging
NaOH	Sodium hydroxide
NBSN	Bromosuccinamide
NMR	Nuclear magnetic resonance
NP-HPLC	Normal-phase high performance liquid chromatography
OGT	O-acetylglucosamine transferase
OGTT	Oral glucose tolerance test
OST	Oligosaccharyltransferase
p-ABA	p-amino benzoic acid
PBS	Phosphate Buffered Saline
PDB	Protein databank
PGC-LC-ESI-MS/MS	Porous graphitized carbon liquid chromatography electrospray ionisation tandem mass spectrometry
PNGase F	Peptide-N-glycosidase F
PPARγ	Peroxisome proliferator-activated receptor γ agonist
PPPM	Predictive, Preventive and Personalised medicine
ROC	Receiver operating curve
RP-HPLC	Reverse-phase high performance liquid chromatography
SBP	Systolic blood pressure
SELDI	Surface-enhanced laser desorption ionization
Ser	Serine
SHS	Suboptimal health status
SLE	Systemic lupus erythematosus
SPE	Solid phase extraction
SPSS	Statistical package for social sciences
SUA	Sulfonylureas
T2DM	Type 2 diabetes mellitus
TaT	Threshing and trimming
TFA	Trifluoroacetic acid
TG	Triglycerides
Thr	Threonine
TNZ	Thiazolidinedione
UDP-N-GlcNAc	Diphosphate-*N*-acetylglucosamine
UPLC	Ultra performance liquid chromatography
WHR	Waist-to-hip ratio
WHtR	Waist-height-ratio
WAX	Weak anion exchange
WAX-HPLC	Weak anion exchange high performance
WHO	World Health Organization

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CHAPTER ONE

1.1 General Introduction

Type II diabetes mellitus (T2DM) (ICD: E11) is a chronic disease that kills many people in both developed and developing countries (The World Health Organisation-WHO, 2011; WHO, 2015). While massive investment has resulted in significant progress in T2DM research in developed countries, similar improvements have not been realised from the African public health perspective. In this thesis, novel, non-invasive screening tools, in conjunction with high-throughput analytical techniques and statistical approaches, were employed to unravel the potential of N-glycosylation profiles as biomarkers for T2DM in a Ghanaian population.

T2DM is a debilitating disorder characterised by progressive and continuous plasma hyperglycaemia (American Diabetes Association, 2014; Kahn, Cooper, & Del Prato, 2014; Shrivastava, Shrivastava, & Ramasamy, 2013). This persisting hyperglycaemia leads to metabolic dysregulation that affects important cells in the kidneys, nerves, heart and blood vessels (American Diabetes Association, 2014). Consequently, T2DM sufferers experience multiple complications including ketoacidosis, urinary tract infections, hyperosmolar non-ketotic coma and stroke, as well as ophthalmic defects (Demirci et al., 2015; Nitzan et al., 2015). Taken together, these complications result in increased hospitalisation (Lim et al., 2013), decreased quality of life (Schofield et al., 2017) and increased mortality (Bao et al., 2017).

Thus far, medical practice has evolved and it is now widely known that hyperglycaemia can be controlled with dietary modifications and changing lifestyles (Shrivastava et al., 2013). However, when these attempts fail to lower hyperglycaemia, parenteral or oral anti-diabetic medications are prescribed (Kahn et al., 2014; Shrivastava et al., 2013). Although these medications have improved clinical outcomes and fundamentally enhanced the lives of T2DM sufferers, they are often associated with adverse effects that lead to withdrawal (Ho et al., 2006). Therefore, targeting and recognising individuals who are at risk of developing T2DM will be more beneficial as such people can be isolated for alternative interventions that may delay the onset of the disease.

Early diagnosis of T2DM may be possible by recognising a reversible, intermediate state, or subclinical disease, hereafter referred to as suboptimal health status (SHS) (Yan et al., 2009). SHS is characterised by poor health, low energy or vitality and

general body weakness (Adua, Roberts, & Wang, 2017; Wang & Yan, 2012; Yan et al., 2014;Wang et al., 2016; Yan et al., 2009; Kupaev et al., 2016). It can be measured using a simple subjective instrument called the SHS-questionnaire (SHSQ-25) (Yan et al., 2009). The SHSQ-25 explores human health from five domains: cardiovascular, fatigue, immune system, digestive system and mental status **(Figure 1.1)** and SHS is scaled based on a cut-off score. Since its introduction, the SHSQ-25 has been successfully applied to chronic disease screening across different populations Wang & Yan, 2012; Yan et al., 2014;Wang et al., 2016; Yan et al., 2009; Kupaev et al., 2016) but this is the first instance of this tool being applied in a West African population (Adua et al., 2017).

Figure 1.1 SHSQ-25 assesses five components of health. Figure reproduced with permission from Wang et al., (2014).

Alternatively, research has advanced and has led to the introduction of objective biomarkers that can gauge human health, monitor disease progression and response to therapy (Herder, Karakas, & Koenig, 2011; Krämer et al., 2010). Fasting plasma glucose (FPG) and the oral glucose tolerance test (OGTT) were introduced in the early 1970s and are still used to diagnose T2DM, but the results of these tests are affected by fluctuations of daily glucose levels (American Diabetes Association, 2010; Bennett, Guo, & Dharmage, 2007; Kilpatrick & Atkin, 2014). After multiple years of investigation, it was revealed that sugars or sugar phosphates irreversibly bind to the β-N-terminal valine residues of globin chains (Schiff base), then undergoes an Amadori rearrangement into a 1-deoxy-1-N-valyl-fructose and forms a ring structure. This product was later referred to as glycated haemoglobin (HbA1c) (Gillery, 2012). HbA1c was evaluated for clinical

practice and has been used to monitor glycaemic control since the 1980s (Kennedy & Baynes, 1984; Brownlee, Vlassara & Cerami, 1984). In 2010, the American Diabetes Association recommended its use for diagnosing prediabetes and diabetes (Gebel, 2012). Thus far, it has been shown that the HbA1c has better diagnostic potential compared to FPG and OGTT by having a lower biological variability, being analytically stable, reflecting glycaemic control over 2-3 months, and not requiring fasting prior to testing (American Diabetes Association, 2014; Kharroubi & Darwish, 2015). However, HbA1c values can be influenced by carbamylated haemoglobin, haemoglobin variant, haemoglobinopathy or abnormal erythrocyte turnover, folic acid deficiency and vitamin B_{12} deficiency (d'Emden, 2012; Rodbard et al., 2009; Gillery, 2012). Moreover, these traditional tests are not robust enough to detect latent or underlying disorders of glucose regulation or metabolism (Keser et al., 2017). More robust biomarkers are therefore needed not only to complement the existing biomarkers, but also to improve early diagnosis. One such biomarker is complex oligosaccharides (glycans) that bind to proteins in glycosylation (Bieberich, 2014; Taylor & Drickamer, 2011).

In fact, studies have established that alteration in profiles of complex sugars (N-glycan) are potential biomarkers for cardiometabolic diseases. Using high performance liquid chromatography (HPLC) and the matrix assisted laser desorption ionisation time of flight (MALDI-TOF) technique, a study reported altered N-glycans in 16 T2DM patients and 16 diabetic (db/db*)* mice (Itoh et al., 2007). However, this study could identify only one N-glycan trait that was associated with T2DM (i.e. α-1, 6-fucosylation) and the sample size was too small to reach statistical significance. Testa et al., (2015) performed N-glycan analysis using DNA sequencer-aided fluorophore-assisted carbohydrate electrophophoresis (DSA-FACE) and found an alteration in N-glycan profiles in T2DM patients compared to controls. However, the DSA-FACE technique yielded only 10 N-glycan peaks and therefore did not provide adequate structural N-glycan characterisation and quantification. In 2011, Lu et al., (2011) applied hydrophilic interaction chromatography (HILIC) and weak anion exchange (WAX) HPLC to detect 10 N-glycan traits that were associated with components of metabolic syndrome (MetS) in Chinese Han and Croatian populations. Five years later, McLachlan (2016) applied the same technique to detect 21 N-glycan traits that were associated with MetS in individuals from Orkney Islands (UK). However, both studies employed a technique that allowed for the detection of 24 N-glycan peaks, only included few MetS components, and did not include markers of liver function.

Recently, another study showed that higher branching, decreased agalactosylated and monogalactosylated, decreased neutral and increased trisialylated N-glycans were associated with T2DM risk in a Croatian population. However, this study failed to adjust for multiple confounders and did not cover other possible risk factors such as lipid levels, waist size, liver enzymes (proteins) and renal biomarkers (Keser et al., 2017). Further, Lemmers et al., (2017) also reported that 5 IgG N-glycans and 13 derived traits were associated with T2DM. However, this study was restricted to only plasma IgG, where IgG only represents a fraction of the total protein in the blood. Hence, total human plasma N-glycome profiling would give a better prediction (Lemmers et al., 2017). Moreover, after reviewing the literature, it became obvious that N-glycosylation studies have largely been conducted among Chinese and Caucasian populations (Lu et al., 2011; McLachlan et al., 2016) and none have thus far, been documented among West African populations. In the translation of the body of scientific knowledge from the Ghanaian perspective, it is argued in this thesis that N-glycosylation profiles among T2DM and healthy controls would be different considering the genetic and environmental factors that contribute to the disease and pharmacotherapies of the disease.

1.2 Thesis aims

In accordance with the limitations of previous studies, and to fill in the gaps in knowledge highlighted above, this thesis is structured as a series of five related studies **(Figure 1.2)** or publications, with each study/publication satisfying a particular aim. These aims are:

1. To examine SHS in a Ghanaian population and, in parallel, examine the anthropometric, clinical and biochemical parameters among T2DM patients.
2. To explore N-glycosylation profiles as risk biomarkers for SHS and MetS.
3. To profile cardio-metabolic risk factors and explore medication utilisation among T2DM patients.
4. To perform high-throughput profiling of whole plasma N-glycans in T2DM patients and healthy individuals.
5. To longitudinally examine N-glycosylation profiles of T2DM from baseline to follow-up.

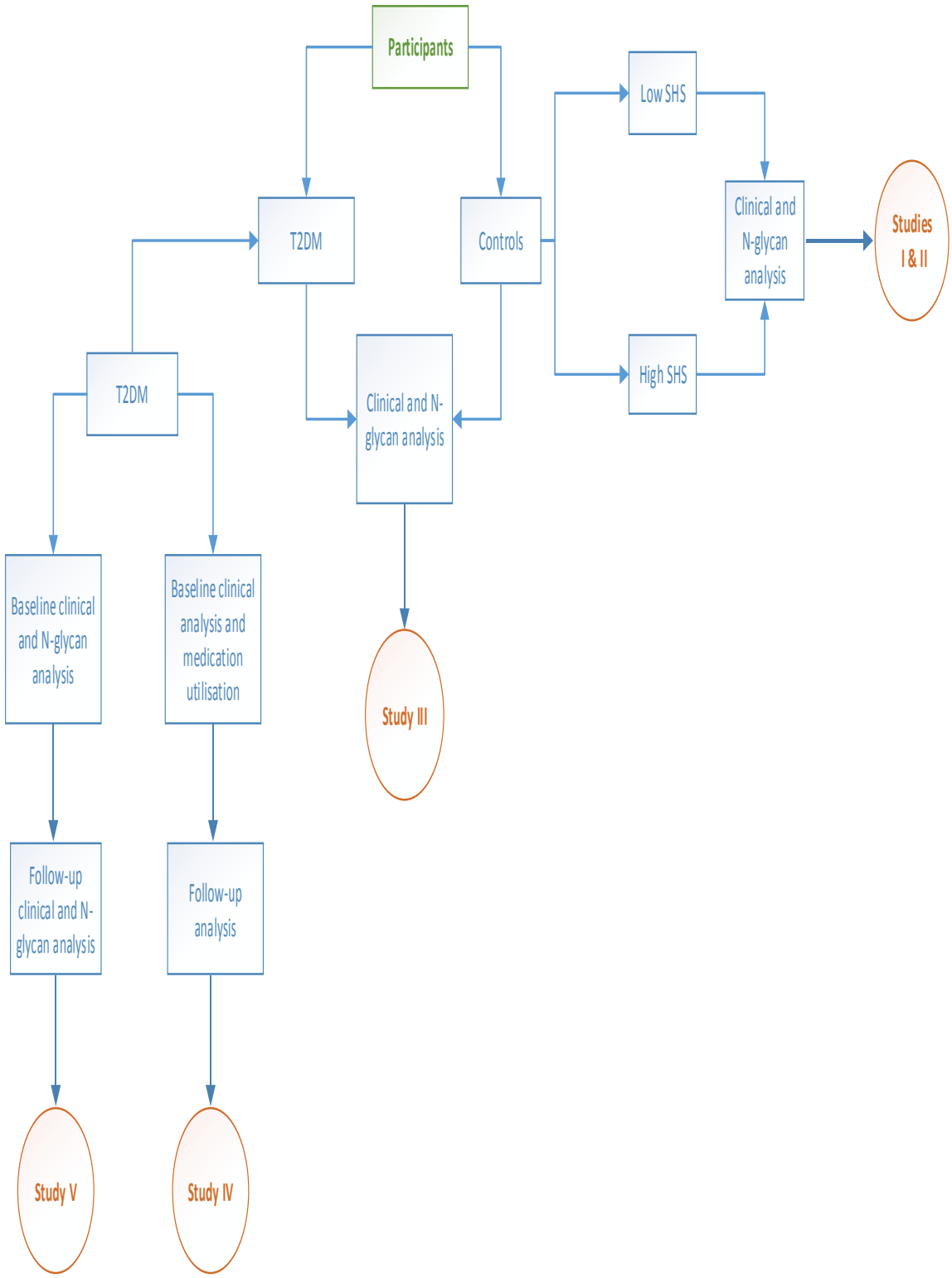

Figure 1.2 an overview of this thesis. The thesis is structured in the form of related studies. **Studies I & II** are cross-sectional, **Study III** is an age-gender matched case-control and **Studies IV & V** are longitudinal.

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CHAPTER TWO

Prelude

Having provided a brief introduction of the thesis and the study aims in **Chapter One**, the **Second Chapter** provides a review of T2DM, highlighting the epidemiology, pathophysiology, genetic and environmental factors that contribute to the disease and pharmacotherapies of the disease.

Literature Review

2.1 Type II Diabetes Mellitus

2.1.1 Epidemiology

Diabetes mellitus (DM) is a disease that may have existed more than two centuries ago and could kill within weeks or months of diagnosis (Polonsky, 2012). After two centuries, the disease can now be managed with medication and lifestyle changes, thereby increasing longevity (Polonsky, 2012). Unfortunately, the route to cure the disease has been slow despite major advances in research and, in fact, the disease is still associated with a significant reduction in life expectancy (Polonsky, 2012). According to the WHO, DM killed 2.2 million people in 2012, and affected 422 million people in 2014 (WHO, 2015). The WHO further states that the disease took 1.6 million lives in 2015 and projected that by 2030 it will be the seventh major cause of adult death worldwide (WHO, 2015). T2DM is the predominant type of DM and it was more prevalent in the developed or high-income countries (Esposito, Kastorini, Panagiotakos, & Giugliano, 2010; Mokdad et al., 2003; Zou et al., 2017). However, recent statistics show that T2DM has reached epidemic levels in low- to middle-income countries (LMIC) and it is even projected that, in the next few decades, two thirds of all DM cases will be found in these countries (Doherty et al., 2014). Sub Saharan Africa (SSA) remains one of the most affected, as an estimated 19.8 million adults above 20 years had T2DM in 2013 while 41.5 million cases are expected by 2030 (Peer et al., 2014). In parallel, an estimated 46% of individuals with T2DM are yet to be diagnosed (Peer et al., 2014) and this is partly due to ageing, such as in the Ghanaian population (Mba, 2010). In Ghana, the disease affected 302,000 adults in 2000 and this is projected to reach 815,000 by 2030 (Guariguata, 2014). Another study also estimated that up to 6% of the adult population in Ghana has DM (Danquah et al., 2012).

2.1.2 Pathophysiology of T2DM

Feedback Regulation of Glucose Metabolism

Under normal conditions, blood glucose levels are maintained via a cross communication involving insulin secretion and tissue sensitivity to insulin (Kahn, Cooper, & Del Prato, 2014). Upon pancreatic β cell stimulation, insulin is released from the islet cells to regulate glucose uptake by insulin sensitive tissues (Kahn et al., 2014). In turn, glucose stimulates the production of insulin from the β cells. However, under abnormal conditions such as T2DM, two main defects occur that disrupt the homeostatic mechanism. These are 1) impaired insulin secretion due to progressive loss of β cell function and, 2) impaired insulin action because of insulin resistance. Combined, these defects lead to decreased glucose uptake by muscle cells, decreased hepatic glucose output and decreased triglyceride uptake by fat cells. To compensate for this defect, the β cells continue to secret insulin in order to maintain normal glucose levels. However, when the β cells are unable to meet this demand, plasma glucose levels rise and lead to impaired glucose levels **(Figure 2.1)** (Cornell, 2015; Epstein, Shepherd, & Kahn, 1999; Kasuga, 2006; Kharroubi & Darwish, 2015; Mellitus, 2005).

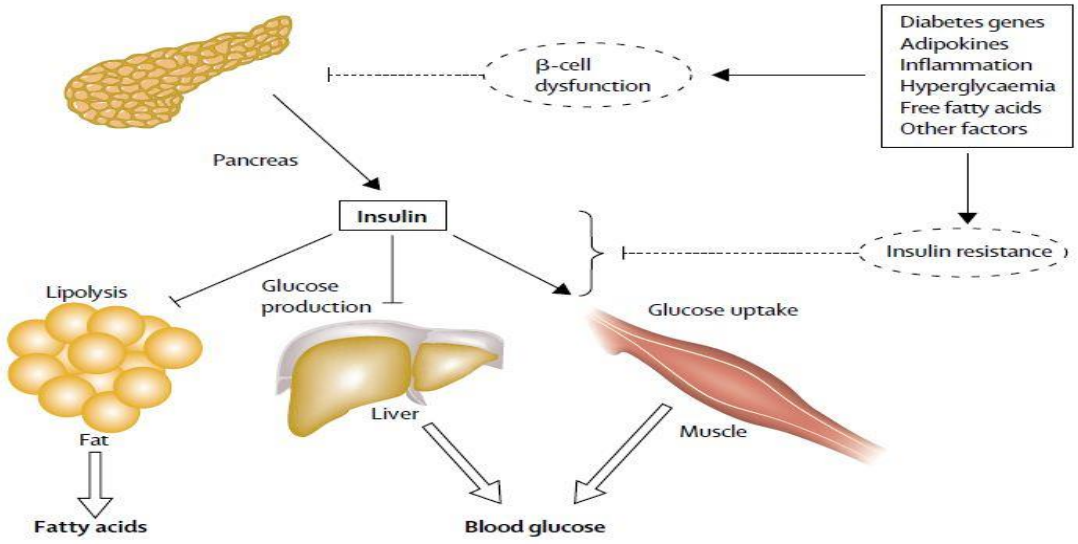

Figure 2.1 Pathophysiology of T2DM. Under normal conditions, insulin is secreted from the β cells of the pancreas to regulate glucose by the liver, promotes muscle glucose uptake and blocks lipolysis (breakdown of lipids into fatty acids). However, in T2DM, insulin secretion is reduced due to progressive loss of β cell function and concurrently, there is impaired insulin action on tissues because of insulin resistance. These effects lead to increased production of fatty acids and blood glucose. Figure reproduced from Stumvoll, Goldstein & van Haeften, 2005.

Insulin Signalling

Insulin mediates the metabolism of carbohydrates, lipids and proteins through a complex signaling cascade. This begins with the binding of insulin to a tyrosine kinase receptor leading to the phosphorylation of insulin receptor substrates 1 and 2 (IRS 1 and IRS 2) (Epstein et al., 1999; Kharroubi & Darwish, 2015; Saltiel & Pessin, 2002). The binding of IRS 1 or IRS 2 to the insulin receptor stimulates phosphoinositol 3-kinase (PI3K) which leads to the recruitment of phosphatidylinositol (4, 5)-bisphosphate (PIP2) and phosphoinositol 3, 4, 5-triphosphate (PIP3) (Gual, Le Marchand-Brustel, & Tanti, 2005; Saltiel & Pessin, 2002). PIP3 binds to Ser/Thr (Akt) or protein kinase B (PKB) that is activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1). Adenosine monophosphate (AMP) then binds, activates and induces a conformational change in adenosine monophosphate-activated protein kinase (AMPK). The activated AMP-K then phosphorylates and activates glucose transporter 4 (GLUT-4). GLUT-4 is a member of the integral membrane glycoprotein family that applies facilitative diffusion to transport saccharides through the cell membrane to generate energy (Ohtsubo et al., 2005; Center, 2000; Epstein et al., 1999) (**Figure 2.2**). Because this is a regulated process, it can be argued that a defect in the signalling cascade can lead to glucose intolerance and insulin resistance. However, it should be noted that T2DM is a multifactorial disease arising from an interplay between environmental and genetic factors.

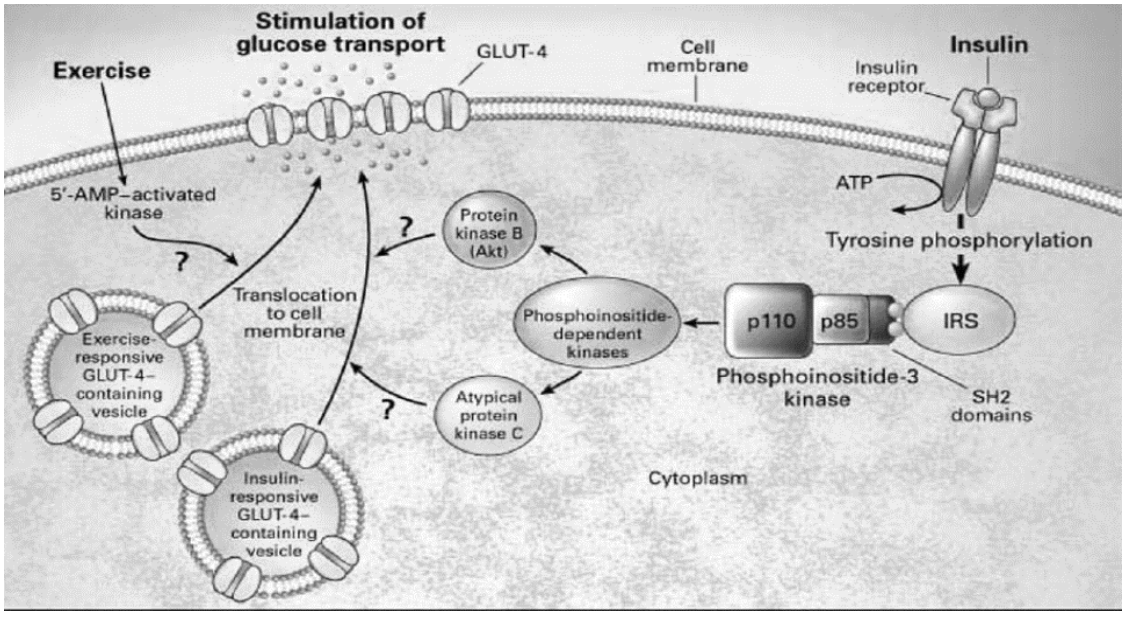

Figure 2.2 Insulin signalling pathway. The insulin receptor is a tyrosine kinase, which phosphorylates IRS 1 and IRS 2. This leads to the activation of PI3K and PDK. Activated PDK 1 can stimulate PKB/AKT or PKC. This effect stimulates the translocation of GLUT 4 to the cell membrane to promote glucose uptake. Exercises tend to influence AMPK, which stimulate the translocation of GLU 4 to the cell membrane (Epstein et al., 1999).

2.1.3 Environmental Factors

T2DM development is largely attributed to dietary and lifestyle habits (American Diabetes Association, 2014; Doherty et al., 2014; Esposito et al., 2008; Frank et al., 2014; Nolan, Damm, & Prentki, 2011). Generally, consumption of foods such as vegetables, fruit, poultry, whole grains, cereal fibre and polyunsaturated fats are associated with decreased risk of T2DM, whereas excess intake of red meat, refined carbohydrates, fried or processed foods and sweets or sugar-sweetened beverages are associated with increased risk of T2DM (Esposito et al., 2008; Frank et al., 2014; Yu et al., 2011). Moreover, consumption of foods that are deficient in micronutrients such as vitamin B12, vitamin D and folic acids increase the risk of T2DM (Pflipsen et al., 2009; De, 2010). Conversely, increased energy expenditure through regular exercise or physical activity reduces the risk of developing T2DM (Phillips, 2017). In fact, studies have shown that 30 minutes of brisk walking per day decreases T2DM risk by 34%, whereas a sedentary lifestyle, including prolonged watching of TV and prolonged sitting at work, was associated with a higher risk (Hu, 2011; Willi et al., 2007). In addition, T2DM risk is fuelled by cigarette smoking and excessive alcohol consumption (Willi et al., 2007). One study has even shown that smoking increases T2DM risk by 45% while heavy alcohol intake leads to impaired glucose metabolism, liver dysfunction and consequently, impaired glucose tolerance (Hu, 2011). Also, depression (Mezuk et al., 2008), sleep deprivation (Shaw et al., 2008) and consistent antidepressant use (Kivimäki et al., 2010) have been implicated in T2DM development. Further, endocrine disrupting chemicals including pesticides, organic pollutants, and toxins contribute to T2DM development (Brook et al., 2010; Chen, Magliano, & Zimmet, 2012; Krämer et al., 2010). It is worthwhile mentioning that developmental reprogramming (poor intrauterine environment with associated fetal growth restriction) contributes to the onset of T2DM (Fernandez-Twinn and Ozane, 2006).

2.1.4 Genetic Susceptibility Genes for T2DM

T2DM is largely a consequence of genetic factors and different approaches have been employed to reveal genetic susceptibility genes. Until recently, genetic mapping for T2DM was mainly by genetic linkage analysis and candidate gene association studies, both of which were limited by small sample size or small pedigree and focus on limited

genes (Grarup, Sandholt, Hansen, & Pedersen, 2014). However, in the past decade, advances in array-based genotyping have made it possible for complete scans of genetic variations in individuals to identify variants that are associated with a particular trait, otherwise referred to as genome wide association studies (GWAS) (Long et al., 2012). The GWAS have not only overcome the shortcomings of genetic linkage analysis and candidate gene association studies but have also provided a better understanding of how genetic defects are associated with insulin resistance and T2DM. Thus far, GWAS have enabled the identification of 176 genomic loci that are associated with metabolic traits and phenotypes (Grarup et al., 2014). Below are a few of the widely reported loci:

1. T2DM-Wolfram syndrome 1 (WFS 1), hepatocyte nuclear factor 1-beta (HNF 1B), insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2), transcription factor 7-like 2 (TCF7L2), solute carrier family 30 (Zinc Transporter), ankyrin 1 (ANK 1), Potassium Voltage-Gated Channel Subfamily J Member 11 (KCNJ11), cell division cycle 123 (CDC 123), member 8 (SLC30A8), JAZF zinc finger 1 (JAZF 1), melatonin receptor 1B (MTNR1B), and haematopoietically expressed homeobox (HHEX).
2. Insulin-peroxisome proliferator-activated receptor gamma (PPARγ), insulin receptor substrate 1 and 2 (IRS 1 and 2), and krupel like factor 14 (KLF).
3. BMI- cyclin dependent kinase 5 regulatory subunit associated protein like- 1 (CDKAL1), fat mass and obesity associated (FTO), SEC16 homolog B (SEC16B), proprotein convertase subtilisin/kexin type 1 (PCSK1), protein kinase D1 (PRKD1), neuronal growth regular 1 precursor (NEGR1).
4. Fasting glucose- purinergic receptor P2X 2 (P2rX2), DNL-type zinc finger (DNLZ), sine oculis-related homeobox 3 (SIX3), DNA topoisomerase 1 (TOP 1), tryptophanyl-tRNA synthetase (WARS).
5. WHR-WARS2, zinc and ring finger 3 (ZNR F 3), homeobox C13 (HOXC13).

It should be noted that T2DM susceptibility genes differ among populations. For example, single nucleotide polymorphism (SNP) r864745 in JAZF 1 is associated with T2DM among European populations (Hu et al., 2009; Zeggini et al., 2008). Similarly, r7754840 in cyclin dependent kinase A like 1 (CDKAL 1) is associated with T2DM from Swedes and Finns. In addition to CDKAL 1, SLC30A8, HHEX, CDKN2A/B, IGF2BP2, FTO and WFS1 are associated with T2DM in Koreans, Chinese and Pima Indians (Hu et

al., 2009; Lee et al., 2008; Rong et al., 2008; Saxena et al., 2007), whereas C2CD4A and KCNQ1 is associated with T2DM among Japanese (Unoki et al., 2008; Yamauchi et al., 2010). Until recently, genome-wide mapping studies in Africa were scarce. The earliest of such studies was the African American Diabetes Mellitus study (AADMS) (Rotimi et al., 2004) involving Ghanaians and Nigerians. In this study, chromosomal locus 20q13.3 was identified as strongly linked with T2DM, a link previously found among non-African populations (Rotimi et al., 2004; Rotimi et al., 2001). Other studies have shown that calpain 10 (CAPN10), PCSK1 and glycoprotein 2 (GP2) were associated with T2DM in Africa and Asia (Chen et al., 2005; Grarup et al., 2014). Further, a genetic risk map of allelic frequencies of 16 variants in 51 ethnic groups in Africa showed that Africans are more genetically susceptible to T2DM than other populations (Tekola-Ayele, Adeyemo, & Rotimi, 2013). Overall, these genetic discoveries could guide T2DM management by providing clues for pharmacological targets and enable precise identification or characterisation for therapeutic interventions for high risk individuals (McCarthy, 2010)

2.1.5 Metabolic Syndrome and Prediabetes

Metabolic syndrome (MetS) is the concurrence of central obesity, dysregulated glucose and insulin metabolism, and hypertension. According to the National Cholesterol Education Program (NCEP) - Adult Treatment Panel ATP III guidelines (NCEP, 2002; Cleeman, 2001), an individual is diagnosed as having MetS when he/she meets the following criteria 1) systolic blood pressure (SBP) ≥ 130 mmHg and/or diastolic blood pressure (DBP) ≥ 85 mmHg, 2) FPG ≥ 100 mg/dL (5.55 mmol/L), 3) waist measurement of ≥102 cm in men and ≥ 88 cm in women; 4) triglyceride (TG) ≥150 mg/dL (1.7 mmol/L) or the use of any lipid controlling medication; or 5) high density lipoprotein cholesterol (HDL-c) of <40 mg/dL (1.0 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women.

Over the past four decades, MetS has received worldwide recognition mainly because of its association with overt diabetes or prediabetes (Wellen & Hotamisligil, 2005; Wilson et al., 2005; Morrison, Friedman, Wang & Glueck, 2008). Prediabetes is an intermediate stage characterised by hyperglycaemia below the level considered to be diagnosed as T2DM but has the likelihood to progress to T2DM (Buysschaert & Bergman, 2011; Kharroubi & Darwish, 2015). For many years, the determination of this intermediate state was via blood glucose measures such as the FPG) and OGTT tests (Sacks et al., 2011). Prediabetes is established when FPG levels are between 100 mg/dl (5.6mmol) to 125

mg/dL (6.94 mmol/l) or impaired glucose tolerance (IGT) is between 140 mg/dL (7.8 mmol/l) to 199 mg/dL (11.0 mmol/l) or HbA1c ≥ 6% (126 mg/dL; 7 mmol/l) but ≤ 6.5% (8.0 mmol/l; 138 mg/dL) (American Diabetes Association, 2014; Buysschaert & Bergman, 2011; Kharroubi & Darwish, 2015). T2DM diagnosis is as follows: plasma glucose after 2hr OGTT ≥ 200 mg/dl (11.1 mmol/l), FPG ≥ 126 mg/dl (7.0 mmol/l) or random FPG of ≥ 200 mg/dL and HbA1c ≥ 6.5%. If any one of these criteria is met, confirmatory tests are performed to establish T2DM (American Diabetes Association, 2014; Sacks et al., 2011).

2.1.6 Pharmacotherapy

Since the description of T2DM many years ago, intense research has led to the discovery of parenteral and oral medications to manage and treat the disease (Kahn et al., 2014). These medications belong to distinct categories based on their modes of action:

1. Biguanides: These are oral hypoglycaemic agents and first line drugs for controlling blood glucose (Saisho, 2015). They regulate blood glucose by reducing hepatic glucose production and inducing muscle glucose uptake by activating AMPK (Saisho, 2015). An example is metformin.
2. Sulfonylureas: These are second line drugs that bind and block adenosine triphosphate (ATP)-sensitive potassium (K) channels in pancreatic cells and stimulate insulin secretion (Rendell, 2004; Stumvoll et al., 2005). Examples of drugs belonging to this class are tolbutamide, tolazamide, glipizide (Glucotrol), glyburide and glibenclamide. Apart from sulfonylureas, other second line drugs include:
3. Glucagon-like receptor agonists (GLP-1): GLP-1 binds to the GLP-1 receptor expressed in pancreatic cells and mediates glucose-dependent insulin secretion and suppresses overproduction of glucagon (Blair & Keating, 2015; St Onge & Miller, 2010). Examples are albiglutide, exenatide, liraglutide and lixisenatide.
4. Dipeptidyl peptidase 4 (DPP4) inhibitors: This class of oral hypoglycaemic agents blocks DPP4, induces incretin (GLP1) levels, has trophic effects on β-cells and decreases glucagon release (Kahn et al., 2014; Stumvoll, Goldstein, & van Haeften, 2005). Examples include Anagliptin, Alogliptin, Sitagliptin, Saxagliptin and Gemigliptin.

5. Peroxisome proliferator-activated receptor γ agonists (PPARγ): This class of drugs act by binding and activating PPARs, receptors that regulate fat storage and glucose metabolism. They reduce inflammatory cytokines that are involved in insulin resistance, lower lipid content in the liver, increase tissue adiponectin and promote insulin sensitisation (Krentz, Bailey, & Melander, 2000). Examples of drugs belonging to this class are pioglitazone, rosiglitazone and lobeglitazone.
6. α-glucosidase inhibitors: These are competitive inhibitors that decrease blood glucose load by prolonging carbohydrate metabolism in the gastrointestinal tract (GIT) and reducing glucose absorption (van de Laar et al., 2005). An example is acarbose.
7. Cannabinoid receptor (CB1) antagonists: These bind and block CB1 receptors leading to a reduction in triglyceride levels and induce weight loss (Scheen et al., 2006). A typical example of such a drug is Rimonabant.

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CHAPTER THREE

Prelude

By focusing on T2DM, **Chapter Two** has given a background to the disease, reporting the prevalence of the disease across the globe and providing insights into the genetic as well as environmental factors that contribute to the diseases' progression. Further, **Chapter Two** has shown that research in T2DM has progressed and has led to the introduction of biomarkers, as well as oral and parenteral medications for control and management of T2DM associated risk factors. However, the pathway to cure still remains far-fetched and therefore, there is the urgent need for additional biomarkers. **Chapter Three** reviews the literature and describes post-genomic biomarkers for chronic diseases such as T2DM. This review has been published in Omics: Journal of Integrative Biology and it can be cited as:

Adua, E., Russell, A., Roberts, P., Wang, Y., Song, M., & Wang, W. (2017). Innovation analysis on postgenomic biomarkers: Glycomics for chronic diseases. OMICS: *Journal of Integrative Biology*, 21(4), 183-196.

Innovation Analysis on Postgenomic Biomarkers: Glycomics for Chronic Diseases

3.1 Abstract

Despite decades of investment in biomarker research, we still do not have robust and optimised biomarkers for many chronic diseases to anticipate clinical outcomes and thus move towards personalised medicine. Biomarker innovations have tended to focus on genomics but the next generation biomarkers from the nascent field of glycomics now offer fresh vistas for innovation in chronic disease biomarkers and systems diagnostics. Glycosylation, regarded as a complex enzymatic process where sugars (glycans) bind to proteins and lipids, affects many human biological functions including cell signalling, adhesion and motility. Notably, and contrary to proteins, glycan biosynthesis does not require a template; rather its final structure is catalysed by a repertoire of enzymes that attach or detach monosaccharides in the glycosylation pathway, making glycomics research more challenging than proteomics or genomics. Yet, given glycans' biological significance, alterations in their processing may be detrimental to human health and offer insights for preventive medicine and wellness interventions. Therefore, studying glycans' structure and understanding their function and molecular interactions in the emerging field of glycomics is key to unravelling the pathogenesis of various common chronic diseases. This review summarises the major concepts in glycomics including glycan release methods, techniques for large-scale glycan analysis and glycoinformatic tools for data handling and storage. In all, this analysis of glycomics offers strategies to build a robust postgenomics innovation roadmap for glycan driven biomarkers as the field is anticipated to mature further and gain greater prominence in the near future.

Key words: Biomarker, glycoinformatics, glycomics, innovation analysis, postgenomics biomarkers

3.2 Introduction

Many chronic diseases still lack innovative diagnostics and biomarkers to anticipate clinical outcomes and thus move towards personalised medicine. Genomics has been a key focus across the biomarker innovation ecosystem but the next generation biomarkers from the nascent field of glycomics now offer fresh vistas for innovation in chronic disease biomarkers and systems diagnostics. Glycosylation, regarded as a complex enzymatic process where sugars (glycans) bind to proteins and lipids, affects many human biological functions including cell signalling, adhesion and motility. Glycans are complex carbohydrates located on the surface of all eukaryotic cells and modify proteins in glycosylation, forming glycoconjugates which thus far, are the most significant post-translational modifications (PTM) (Cummings & Pierce, 2009; Harada, Hirayama, & Suzuki, 2015; Stanley & Cummings, 2009). For the over 19,709 human protein genes known, an estimated 39% are predicted to be either on the intracellular surface or outside of the cell (Gordan Lauc, 2016). Thus, about 7,686 proteins have these glycans bound to them (Apweiler, Hermjakob, & Sharon, 1999; Gordan Lauc, 2016). When attached to proteins, glycans affect all aspects of the protein including function, trafficking, folding and clearance (Helenius & Aebi, 2001; Stanley, 2011; Zoldoš, Horvat, & Lauc, 2013). So far, four protein modifying glycan types have been identified: 1) N-glycans **(Figure 3.1)**; 2) O-glycans; 3) glycosaminoglycan (GAG); and 4) C-glycans (Ajit Varki, 2009; Fuster & Esko, 2005; Hofsteenge et al., 1994; Jensen, Karlsson, Kolarich, & Packer, 2012; Schachter, 2000; Stanley & Cummings, 2009; Weerapana & Imperiali, 2006).

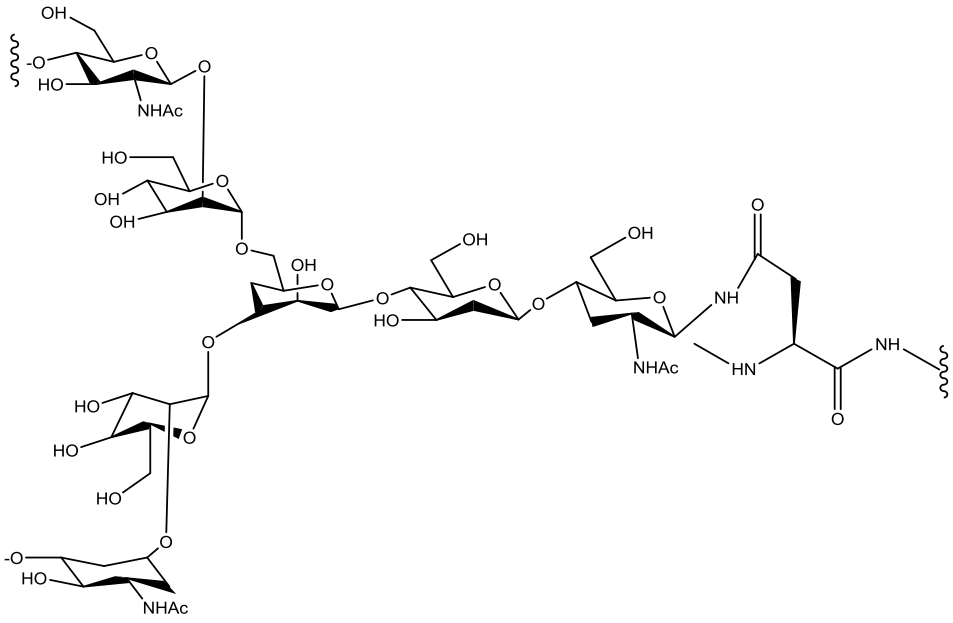

Figure 3.1 N-linked glycan structure, redrawn from Dube and Carolyn, 2005.

3.3 Mechanism of Glycosylation

The mammalian cell is known to contain a repertoire of glycans (Cummings & Pierce, 2009). These glycans comprise chains of monosaccharide units that are linked by α and β-glycosidic bonds (Ajit Varki, 2009; Taylor & Drickamer, 2011). As mentioned earlier, four main types of glycans exist: N-glycans, O-glycans, GAGs and C-glycans. These glycans differ in their core structure, whether they are branching or not, and the recognition sequence, if any, by which they attach (Varki, 2009; Stanley & Cummings, 2009).

N-glycans are the most common and well understood, with an estimated 50%-90% of plasma proteins being N-glycosylated (Bieberich, 2014; Lu et al., 2011; National Research Council (US) Committee on Assessing the Importance and Impact of Glycomics and Glycosciences, 2012). In contrast to proteins, N-glycan biosynthesis is not directly template driven (Brooks, 2009). Instead, a repertoire of enzymes that create, degrade, or modify monosaccharide glycosidic bonds in the endoplasmic reticulum (ER) and the Golgi apparatus determine the final glycan structure (Rini, Esko, & Varki, 2009; Stanley, 2011; Taylor & Drickamer, 2011). In brief, a given oligosaccharide glycan precursor, made up of three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc) monosaccharides ($Glc_3Man_9GIcNAc_2$), is formed in the ER on a lipid dolichol phosphate (Dol-PP) (Rosnoblet et al., 2013; Yan & Lennarz, 2005). The oligosaccharide complex is transferred from the Dol-PP to the N-group of Asn on a given protein, catalysed by oligosaccharyltransferases (OSTs) **(Figure 3.2)** (Yan & Lennarz, 2005). Proteins having the conserved sequence asparagine [(Asn)-X-Serine (Ser)] or [Asn-X-Threonine (Thr)], where X is any amino acid except proline; for glycosylation within the lumen of the ER serve as acceptors (Smith, Ploegh, & Weissman, 2011; Taylor & Drickamer, 2011). In the ER and following different folding states of the newly synthesised glycoprotein, the oligosaccharide precursor undergoes trimming by specific enzymes, known as glycosidases (Rosnoblet et al., 2013). This trimming signals that the glycoprotein is being correctly folded. It is then translocated to the *cis* portion of the Golgi apparatus for further trimming by glycosidases and elongation by glycosyltransferases into different structural types—all N-glycans maintaining the conserved common core G1cNAc2Man3 (Varki, 2009).

Additionally, other modifications, such as *N*-acetylglucosaminylation, fucosylation, sialylation, and galactosylation, occur in the *medial* to *trans* Golgi during maturation (Ohtsubo & Marth, 2006; Schwarz & Aebi, 2011).

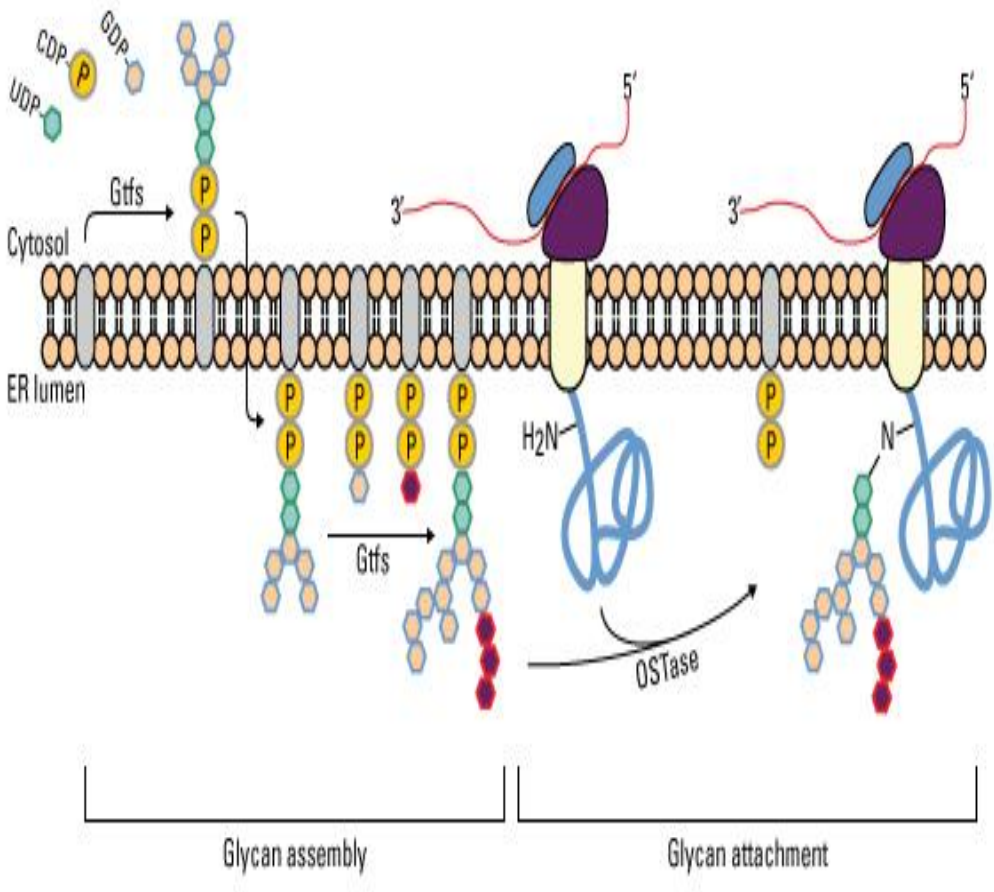

Figure 3.2 Mechanism of N-glycosylation. The process of N-glycosylation begins with the transfer of pre-formed glycans by means of a lipid precursor in the ER. Oligosaccharide-dolichol precursor then binds to the asparagine residue of a nascent protein. The complex undergoes trimming and additional processing in the Golgi. Figure reproduced from http://www.thermofisher.com/au/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/protein-glycosylation.html.

The three main structural types of N-glycans are oligomannose (or high-mannose), complex, and hybrid (Stanley & Cummings, 2009). The high-mannose type, consisting of $Man_{5-9}G1cNAc_2$, is formed after Glc and Man removal, and lacks additional monosaccharide units at the N-glycan periphery (Bieberich, 2014). Complex N-glycans have antennae attached to the core of the structure and can be further subcategorised into bi-antennary, tri-antennary, and tetra-antennary based on the degree of branching (Bieberich, 2014). The branching is formed by the addition of galactose (Gal) and sialic acid monosaccharides to the G1cNAc by galactosyltransferases and sialyltransferases,

respectively (Stanley & Cummings, 2009; Taylor & Drickamer, 2011). Combined structural features from the high-mannose and complex types form the hybrid structural type of N-glycans (Stanley, 2011). Usually, the hybrid N-glycans have only Man residues on the α-1, 6-arm of the core, with one or two branches on the α-1, 3-arm **(Figure 3.3)**. The complex and hybrid types predominantly have a fucose (Fuc) group attached either to a branch or the G1cNAc side chain, known as fucosylation or core fucosylation, respectively (Stanley & Cummings, 2009).

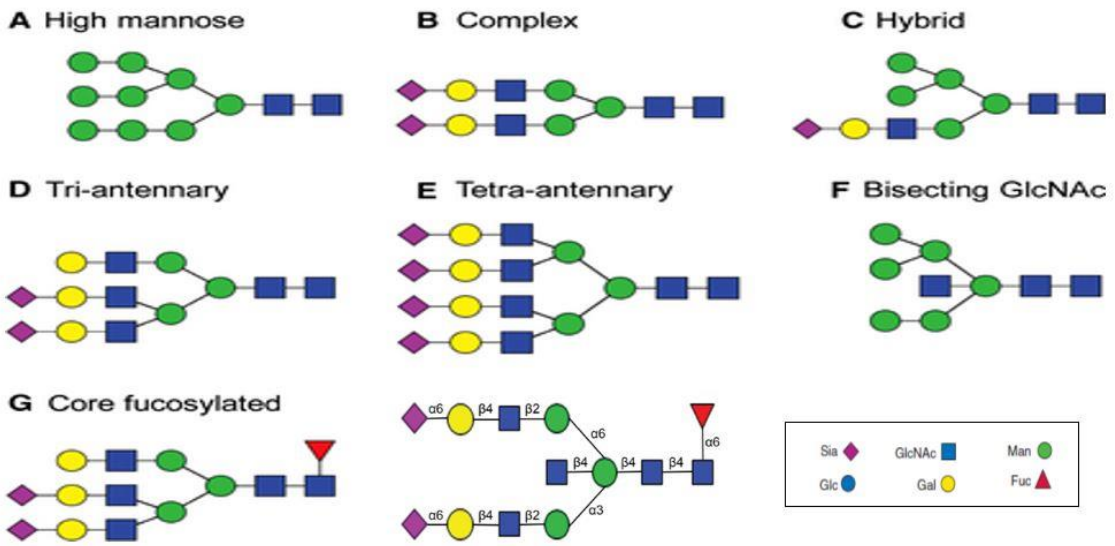

Figure 3.3 Structural types of N-glycans. N-glycans have two core GlcNAcs; having F before the abbreviation represents a core fucose. The two main stereochemical types (orientation) of glycosidic bonds are "α" and "β". The numbers indicate the ring position of the carbon on the saccharide (sugar) which gives rise to the glycosidic bond. For example, a biantennary structure with two GlcNAcs as α-1-2 linked, [3] G1 and [6]G1 indicates that the galactose is on the antenna of the α-1-3 or a α-1-6 mannose. N-glycans can differentiate into bisecting, tri-antennary tetra-antennary (Stanley & Cummings, 2009).

O-glycans are the second most common type of glycan. Unlike N-glycosylation, O-glycosylation is a spontaneous process, akin to tyrosine phosphorylation and occurring in the Golgi apparatus (Dias & Hart, 2007; Lefebvre et al., 2003). The O-glycosylation recognition site is currently unknown (Butkinaree, Park, & Hart, 2010; Christlet & Veluraja, 2001; Schachter, 2000); however, it is a sequential process that involves the attachment of O-linked GlcNAc (O-G1cNAc) to Ser or Thr residues by O-G1cNAc-transferase (OGT). A series of enzymatic steps in the Golgi yields the eight O-glycan cores that are modified by sulfation, acetylation, and fucosylation to form branched structures **(Figure 3.4)** (Butkinaree et al., 2010). O-glycans are similar to N-glycans, as they contain branches extended by monosaccharide units (Alley & Novotny, 2013; Jensen et al., 2012).

Glycans are able to interact with other molecules based on the structural elements

present. GAGs are unbranched polysaccharides which can exist either as part of a proteoglycan or as a free oligosaccharide (hyaluronan) (Fuster & Esko, 2005) **(Figure 3.4)**. GAGs attach to oxygen in Ser and Thr residues at the consensus sequence Ser-Glycine (Gly)-X-Gly, where X is any amino acid (Varki, 2009). Proteoglycans, such as heparan sulfate and chondroitin sulfate, are synthesized via a different pathway; they are more sulfated than O-glycans, but with the same binding specificity. Hyaluronan, a distinct form of GAG, does not bind to lipids or proteins (Fuster & Esko, 2005; Ohtsubo & Marth, 2006) and is involved in cell signaling and tissue homeostasis. In rare cases, C-glycosylation occurs where Man residues are bound to tryptophan (Trp) in the sequence Trp-X-X-Trp through a carbon-carbon bond, where X is any amino acid (Hofsteenge et al., 1994).

Glycans can be involved in both homotypic and heterotypic interactions that form glycolipids (Ohtsubo & Marth, 2006). Glycolipids are also biosynthesized in the ER and the Golgi apparatus. Two types of glycolipids are known, namely glycosphingolipids and glycosylphosphatidylinositol (GPI)-anchors **(Figure 3.4)**. Glycosphingolipids are amphipathic membrane-bound glycoconjugates (e.g., glycocalyx) or secreted molecules within the extracellular matrix, and are important mediators of cell motility and signaling events (Fuster & Esko, 2005). Glycosphingolipid biosynthesis begins with the addition of Glc to ceramide moieties in the ER. Further modification of the Glc-ceramide complex with other Glc residues yields the matured structure in the Golgi. GPI-anchors are formed when glycan chains are attached to phosphatidylinositol **(Figure 3.4)**. GPI-anchors are biosynthesised in the ER and mature in the Golgi. In the Golgi, the GPI-anchor is either modified with phosphatidylethanolamine or GlcNAc molecules before they are complexed with a Gal or sialic acid. Final GPI-anchor structures are then translocated to the plasma membrane (Fuster & Esko, 2005; German et al., 2007).

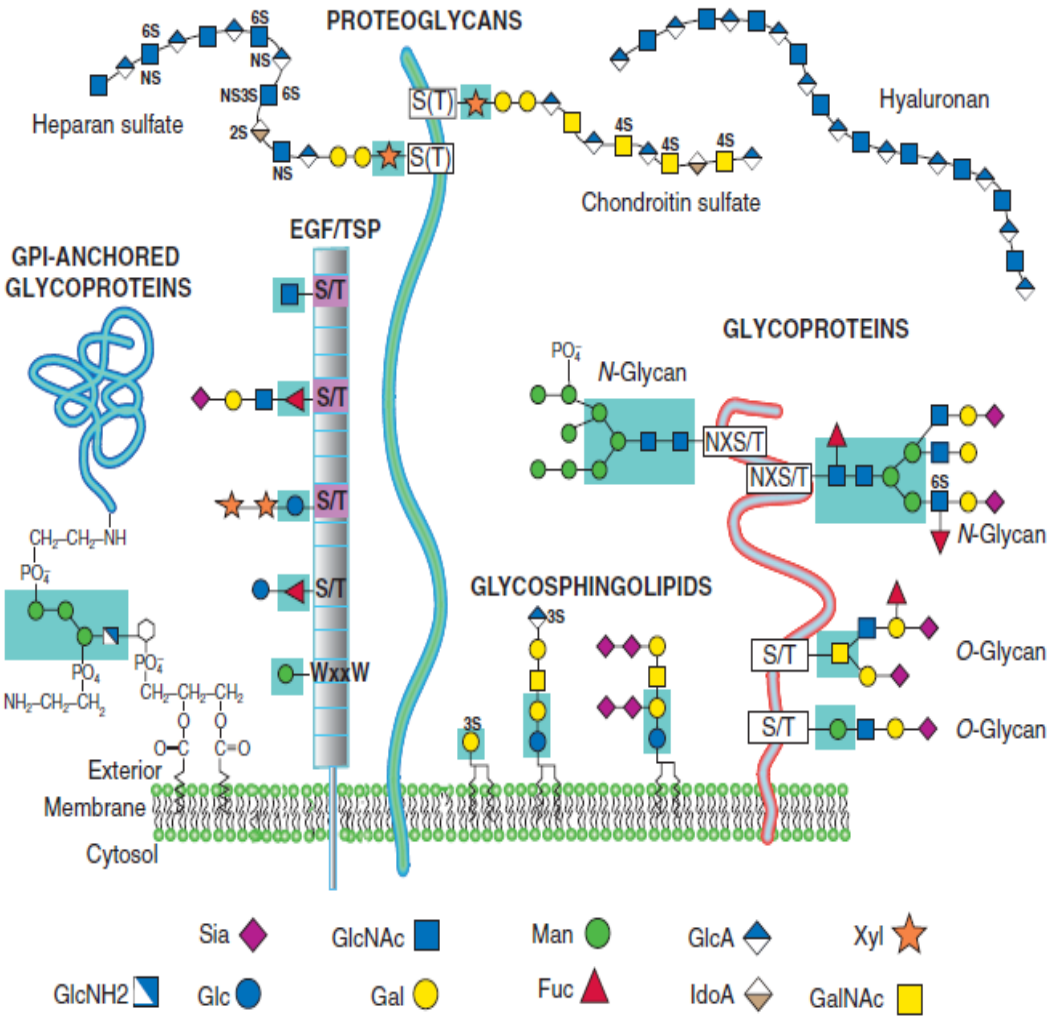

Figure 3.4 Different types of glycans in the cell. The diagram shows the different glycan types in the Golgi apparatus including N-glycans, O-glycans, two examples of proteoglycans (heparin sulphate and chondroitin), and membrane bound glycans such as glycocalyx, glycosphingolipids and GPI-anchors (Fuster & Esko, 2005). Abbreviations: glucose, Glu, Mannose-Man, N-acetylgalactosamine-GaINAc-, N-acetylglucosamine-GIcNAc, fucose-Fuc, Glucuronic acid-GIcA, Sialic acid- Sia Xylose-Xyl. Diagram obtained from Golgi glycosylation, with permission from Cold Spring Habour publishers.

N-glycans' role in cellular processes has been extensively reviewed, including cell communication, signalling, adhesion, motility and host pathogen interactions (Brooks, 2009; Helenius & Aebi, 2001; Jianguo Gu, 2012; Ohtsubo & Marth, 2006). N-glycans are stable over extended periods and therefore a change in their structure can be as a consequence of an environmental or pathophysiological condition (Gornik et al., 2009; Lu et al., 2011). Perhaps this is the reason why aberrant N-glycans are linked to several chronic diseases such as cancers (Arnold, Saldova, Hamid, & Rudd, 2008; Lauc et al., 2013; Saldova, Arnold, & Rudd, 2011; Wang, 2013), hypertension (Wang et al., 2016), metabolic syndrome (MetS) (Lu et al., 2011; McLachlan et al., 2016), diabetes (Testa et al., 2015), systemic lupus erythematosus (SLE) (Vučković et al., 2015), rheumatoid

arthritis (Sebastian et al., 2016), Parkinson's disease (Russell et al., 2017) and congenital disorders of glycosylation (CDG) (Grünewald, Matthijs, & Jaeken, 2002). Given the critical role of N-glycans in human physiology, high-throughput techniques and glycoinformatic tools that fine-tune structural determination and analyses are mandatory.

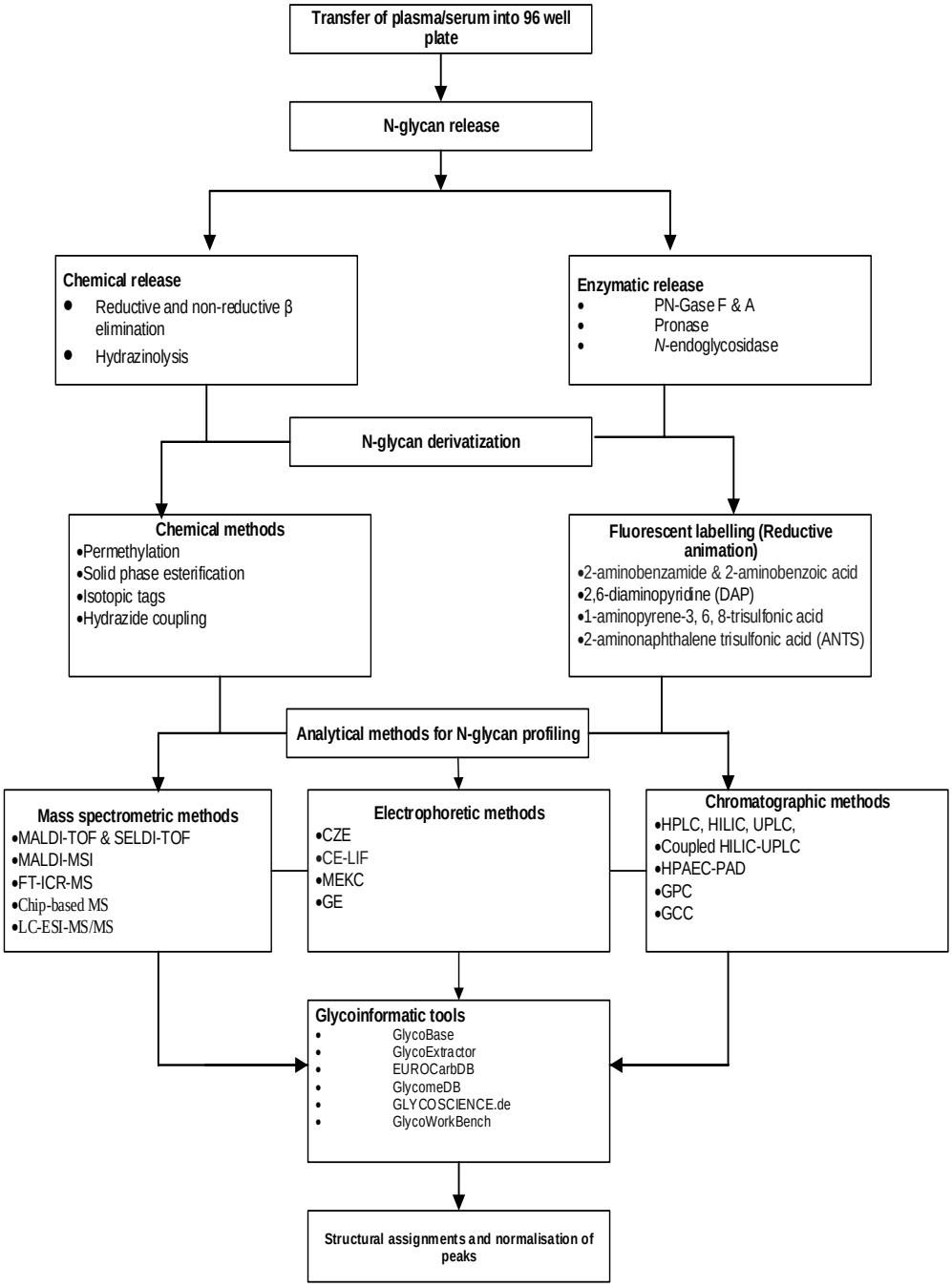

Figure 3.5 Schematic representation of N-glycan profiling. N-glycans can be released from proteins either by chemical or enzymatic approaches. N-glycans are then derivatised by chemical methods or by flourescent labelling. Different analytical approaches are then employed for charactisation and separation. Glycoinformatic tools are employed to aid experimental data storage, N-glycan structure annotation and interpretation into computer-readable formats that can be easily accessed by glycobiologists.

3.4 Methods of N-glycan Release

N-glycans exist as protein or lipid conjugates (Helenius & Aebi, 2001). Therefore, in order to analyse and determine their structural features, they are liberated from their bound proteins or lipids in a process termed deglycosylation (Hägglund et al., 2007). In the past, deglycosylation was a challenging task to accomplish but recent advances in deglycosylation methods have made it possible to release N-glycans with less difficulty. The choice of a particular method depends on the type of glycosylation, the composition and the amount of sample to be analysed (An et al., 2009; An et al., 2003). Generally, N-glycans are released either by enzymatic or chemical means (Alley & Novotny, 2013; Hägglund et al., 2007; Merry & Astrautsova, 2003; Yang et al., 2016) **(Figure 3.5)**. In the enzymatic approach, the enzyme mostly used is peptide-*N*-glycosidase (PNGase) F or *N*-glycanase, which cleaves N-glycans with efficiency (Hägglund et al., 2007; Krishnamoorthy & Mahal, 2009; Mechref, 2011). Here, N-glycans are first cleaved into their 1-amino forms followed by a deamination process that converts Asn to aspartic acid (Mechref, 2011). However, α-1, 3-linked fucose modified proteins are cleaved with PNGase A since it leaves the fucose group intact, thereby enabling the identification of complex and hybrid forms (Alley & Novotny, 2013; Hägglund et al., 2007; Mechref, 2011). For glycoproteins that are resistant to both PNGase F and A, pronase, a collection of proteolytic enzymes from *Streptomyces griseus*, is preferred. Pronase is only limited by its long incubation period, which is now known to be reduced after immobilising on solid supports (Alley & Novotny, 2013). By way of enhancing pronase activity, Song et al., (2014) recently described the threshing and trimming method (TaT) where glycoproteins in tissues were first digested (threshed) with pronase into small peptides and glycoamino acids. Glycoamino acids were then trimmed with *N*-bromosuccinamide (NBS) to yield free N-glycans. TaT has several advantages: 1) because the method requires mild conditions using non-toxic reagents, both labile groups and N-glycan reducing ends are unaffected; 2) compared to other chemical methods, it is less expensive, making it a robust alternative for large-scale deglycosylation (Song et al., 2014); and 3) like PNGase F, TaT is also effective at cleaving α-1-3-linked fucose modified proteins (Song et al., 2014).

Other deglycosylation enzymes are peptide N-endoglycosidase (Endo) H, D, F1, F2 and F3 (Merry & Astrautsova, 2003). While Endo H and Endo F1 cleave both high mannose and hybrid N-glycans, Endo D is specific for complex N-glycans (Tarentino,

Trimble, & Plummer Jr, 1989). Endo F2 and Endo F3 cleave biantennary and triantennary N-glycans, respectively, while tetraantennary N-glycans are cleaved by Glycosamidases (Hägglund et al., 2007; Merry & Astrautsova, 2003). N-glycans are released chemically by β-elimination (reductive and non-reductive). Here, N-glycans are cleaved from glycoproteins in an alkaline medium that converts monosaccharides at the reducing end to alditol or GalNAc-ol (Cummings & Pierce, 2014; Roth, Yehezkel, & Khalaila, 2012). The non-reductive β-elimination method is sometimes preferred because the reducing end aldehyde is left intact for fluorescent labelling and the presence of strong reducing agents minimises peeling reactions, thus making it useful for large-scale N-glycan release (Cummings & Pierce, 2014). Another means of releasing N-glycans is by hydrazinolysis under high temperatures (95^{o}C) (Brooks, 2009). Hydrazinolysis entails complexing glycoproteins with hydrazines immobilised on beads, resulting in the breakdown of the protein portion of a glycoprotein (Lazar, Lee, & Lazar, 2013). However, hydrazinolysis is rarely used for N-glycan release because the reagents are toxic and destroy important labile groups (Lazar et al., 2013; Song et al., 2014). Additionally, the reagents interfere with the bonds linking glycans to Asn, making it difficult to determine the glycosylation site. Furthermore, integrity of the N-glycans may be compromised since some reagents are incorporated into the terminus of their structure (Cummings & Pierce, 2014).

Overall, the described deglycosylation methods are effective and once detached; N-glycans are ready for characterization, purification and structural assignment.

3.5 N-glycan Derivatising Methods

Considering N-glycans are non-UV absorbing biomolecules, detection using various chromatographic methods is often difficult. At the same time, several hydroxylic side chains disallow sensitive detection using mass spectrometry (MS). Thus, they are usually derivatised to enhance detection (Ruhaak et al., 2010). Two main derivatisation approaches are generally employed; permethylation (chemical) and reductive animation. Selecting a particular approach is in part dependent on which analytical technique is to be used. While permethylation is often required prior to MS (Hung et al., 2012), reductive animation is usually preferred during chromatographic separations (Zaia, 2008) **(Figure 3.5)**.

In 1960, Hakomori described permethylation, which involves replacing hydrogen groups bonded to oxygen and nitrogen in the presence of dimethylsulfoxide, methyliodide and sodium hydroxide (NaOH) to form hydrophobic derivatives (Hakomori, 1964). When

compared to native N-glycans, permethylated ones are more stable, less solvated and have higher surface activity, resulting in increased ion abundance in MS (Alley Jr & Novotny, 2013; Walker, 2011; Zaia, 2008). In addition, permethylation allows uniform ionization and simultaneous measurement of both neutral and acidic N-glycans. Further, the ease of predicting fragments of permethylated N-glycans as compared to the native ones facilitates structural assignment (Ruhaak et al., 2011). Although traditional permethylation has improved MS analysis, peeling reactions and oxidative degradation due to excess NaOH arise, thereby reducing sensitivity (Furukawa, Fujitani, & Shinohara, 2013; Kang et al., 2005). However, this has been ameliorated by solid-phase permethylation, which employs reduced NaOH in micro spin columns. This new method not only improves permethylation efficiency by limiting peeling reactions and removing excess reagents, but also enhances sample recovery (Desantos-Garcia et al., 2011; Kang et al., 2005). For complex N-glycans, permethylation is often not used because of possible loss of N-acetylneuraminic acid (sialic acid) groups; rather solid-phase esterification (SPE) is preferred. SPE stabilises the sialic acids by converting all carboxylic acid groups to methyl esters. When compared to underivatised N-glycans, esterified N-glycans significantly improve the overall MS sensitivity (Alley & Novotny, 2013).

Due to the lack of fluorescent moieties and chromophores on the free reducing ends of N-glycans, they are derivatised with specific labels or tags by reductive animation. Among the widely used labelling tags are 2-aminobenzamide (2-AB) (Bigge et al., 1995; Watanabe et al., 2000), 2-aminobenzoic acid (2-AA), 2-aminopyridine (2-AP), 2,6-diaminopyridine (DAP)(Xia et al., 2005), 2-aminonaphthalene trisulfonic acid (ANTS) and 1-aminopyrene-3, 6, 8-trisulfonic acid (APTS) (Callewaert et al., 2004). The drawback with this method is the long period for sample analysis because of persistent salt contamination, which necessitates sample clean up. Other isotopic tags that are commonly used are [$^{12}C_6$] or [$^{13}C_6$]-aniline, $^{13}CH_3I$ (Gerardo et al., 2007) and hydrazide coupling (Walker et al., 2011).

Table 3.1. N-glycans as biomarkers for chronic diseases

Type of disease	Technique	Sample	Glycan biomarker	Nature of change
Breast cancer (Radka Saldova et al., 2014a)	UPLC-HILIC, WAX-HPLC	Serum	• Biantennary core fucosylation • Outer-arm fucosylation • Monosialylation • Sialylation • Branching	↓ ↑ ↓ ↑ ↑
Gastric cancer (Liu et al., 2013)	DSA-FACE	Serum	• α-1,3-fucosylated triantennary • Core- α-1,6-fucosylated biantennary • Core- α-1,6-fucosylated bisecting biantennary • Bigalacto- core- α-1,6-fucosylated biantennary	↑ ↑ ↑ ↓
Hepatocellular carcinoma (Fang et al., 2010)	DSA-FACE	Cell line	• α-1, 3-fucosylated triantennary • Biantennary glycans	↑ ↑
Rheumatoid arthritis (Nakagawa et al., 2007)	HPLC, MS	Serum	• Mono-galactosyl bi-antennary N-glycans	↑
Metabolic syndrome (Lu et al., 2011)	HILIC, WAX-HPLC	Plasma	• Core-fucosylation • Triantennary • Monosialylation • Diasialylation • Triasialylation • Biantennary • Trigalactosylation	↓ ↑ ↓ ↑ ↑ ↓ ↑
Type II diabetes (Testa et al., 2015)	DSA-FACE	Serum	• Core- α-1,6-fucosylated biantennary • Digalactosylated biantennary • α-(1,6)-arm mono galactosylation • α-(1,3)-arm mono galactosylation	↓ ↑ ↓ ↓
Type II diabetes (Itoh et al., 2007)	HPLC, MALDI-TOF	Sera	• α-1,6-fucosylation	↑
Systemic lupus erythematosus (Vučković et al., Barrios, 2015)	UPLC	Plasma (IgG)	• Galactosylation of IgG • Sialylation • Core fucose • Bisecting GlcNAc	↓ ↓ ↓ ↓
Rheumatoid arthritis (Nakagawa et al., 2007)	LC-MS	Serum (IgG)	• Mono-galactosyl bi-antennary • Triantennary	↓ ↑

3.6 N-glycosylation in Precision Medicine

As stated earlier, a growing number of disorders have been directly linked to N-glycosylation **(Table 3.1)**. In addition, because genetic polymorphisms are distant from the phenotypes, and the sophisticated nature of gene-gene interactions, glycans may be "intermediate" and "dynamic" biomarkers for risk stratification, diagnosis, and prognosis of diseases (Dube & Bertozzi, 2005; Ohtsubo & Marth, 2006; Russell et al., 2017).

3.6.1 N-glycosylation in Risk Stratification

At least one third of deaths are potentially preventable by reducing the prevalence of known risk factors (e.g., smoking, poor, diet and inadequate physical activity) (Hulsegge et al., 2017; Lim et al., 2013; Mokdad et al., 2003). Assuming that precision medicine is to provide the right treatment to the right patient at the right time, precision public health can be viewed as providing the right intervention to the right population at the right time (Golubnitschaja, Kinkorova, & Costigliola, 2014). More accurate methods for measuring disease, pathogens, exposure, behaviours, and susceptibility could be used to stratify the risk of disease. However, no models are currently available that predict disease prevalence based on glycosylation biomarkers.

3.6.2 N-glycosylation in Diagnosis

Evidence that glycans are altered in many chronic diseases **(Table 3.1)** make it prudent to underscore the power of investigating and utilising N-glycans as disease biomarkers. N-glycans are present in serum and plasma, as well as other tissues and fluids, making them easily accessible (Gornik et al., 2009; Trbojevic-Akmacic, Vilaj, & Lauc, 2016). Characterization of glycan biomarkers relies on the precise identification of the connection between the glycan modification and the disease (Dube & Bertozzi, 2005; Lauc et al., 2016a). This knowledge may then be further applied to pharmacologic agents that could alleviate the clinical and subclinical symptoms of disease (Taniguchi & Kizuka, 2015).

3.6.3 N-glycosylation in Cancer

Glycans are involved in numerous fundamental molecular and cell biology processes in cancer cells including cell signaling and communication, tumor cell dissociation and invasion, cell–matrix interactions, tumor angiogenesis, immune modulation, and metastasis formation. Therefore, glycans may be utilised as potential

cancer biomarkers and provide a set of specific targets for therapeutic intervention (Alley et al., 2012; Fuster & Esko, 2005; Miyoshi, Moriwaki, & Nakagawa, 2008; Taniguchi & Kizuka, 2015; Wang, 2013).

An analysis of 140 N-glycan peaks from the serum of 107 breast cancer patients and 62 healthy controls showed that the former had increased fucosylation and sialylation (Saldova et al., 2014). Based on the analysis of nine structural glycan peaks from 375 people, comprising 247 gastric cancer (GC) patients and 128 healthy controls, there was a decrease in the level of core fucose residues and fucosyltransferase in GC patients compared with controls (Liu et al., 2013). An increase in branched α-1, 3-fucosylated triantennary and biantennary glycans in cell lines is associated with hepatocellular carcinoma (HCC) (Fang et al., 2010; Miyahara et al., 2015) and an increase in α-1, 6-fucosylation of α-fetoprotein is also linked to HCC (Aoyagi, 1995). Levels of α-1, 6-fucosyltransferase are higher in human ovarian serous adenocarcinomas, liver cirrhosis, and hepatomas compared with controls (Miyoshi et al., 1999). Recently, Ren et al., (2016) performed for the first time a large-scale, multi-institute study to assess the quantitative changes of IgG glycosylation in 12 types of cancers and non-malignant controls, and found that the Gal-ratio of IgG could distinguish these 12 types of cancers from non-cancer controls, as well as early-stage cancers from non-cancer controls.

3.6.4 N-glycosylation in Inflammatory and Autoimmune Diseases

The N-glycosylation of the immunoglobulin G (IgG) Fc domain mediates the effector function of IgG, and hence IgG glycosylation disorders are implicated in inflammatory and autoimmune diseases (Lauc et al., 2013; Vučković et al., 2015). A study of 15 rhematoid arthritis (RA) patients and 18 matched controls showed a decrease in monogalactosyl bi-antennary glycans and an increase in triantennary serum glycans of IgG in RA (Itoh et al., 2007; Nakagawa et al., 2007). Analysis of 251 SLE patients and 252 healthy controls in Latin America as well as replication cohorts from Trinidad (108 cases and 193 controls) and China (107 cases and 200 controls) has shown that there is a decrease in sialylation and galactosylation of plasma IgG among systemic lupus erythematosus (SLE) patients. Similarly, these patients have a decrease of core-fucose and increased bisecting plasma GIcNAc in SLE (Vučković et al., 2015). An investigation of plasma IgG glycome composition in patients with ulcerative colitis (UC) (n = 507), Crohn's disease (CD) (n = 287), and controls (n = 320) showed that both UC and CD were associated with significantly decreased IgG galactosylation and a significant decrease in

the proportion of sialylated structures in CD (Trbojevic-Akmačić et al., 2015). One study explored the association between IgG glycans and renal function in 3274 individuals from the Twins UK registry, and found 14 glycan traits were associated with renal function in the discovery sample and remained significant after validation (Barrios et al., 2016). Recently, in a clinical-based case-control study comprising 128 Han Chinese patients suffering from chronic RA and 195 gender- and age-matched controls, it was found that IgG glycans might have potential as a putative biomarker for RA in the Han Chinese population, and differs in RA active and remission states (Sebastian et al., 2016).

3.6.5 N-glycosylation in Metabolic Diseases

It has been shown that serum protein α-1, 6-fucosylation was increased in 16 diabetic mice (*db/db*) compared with controls (Itoh et al., 2007). The study further demonstrated that α-1, 6-fucosylated biantennary and bisecting *N*-acetylglucosamine serum glycans were increased in 20 T2DM patients (Itoh et al., 2007). Similarly, 10 analysed N-glycan peaks in 562 T2DM patients and 599 healthy controls, demonstrated that T2DM patients had significant increases in fucosylation and galactosylation of serum proteins (Testa et al., 2015). Previous investigation of the association between core fucosylated plasma glycans and MetS in 212 Chinese Han and 520 Croatian individuals, found 10 N-glycan structures (monosialylated, FUC-C, trisialylated, trigalactosylated (G3), digalactosylated (G2), disialylated (S2), triantennary (TRIA), biantennary agalactosylated (A2), biantennary (BA), and agalactosylated (G0) N-glycans) tended to be associated with MetS components, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), and fasting plasma glucose (FPG) levels (Lu et al., 2011). The association between IgG glycosylation and hypertension in a multiple ethnic cross-sectional study (Chinese Han, Croatian, and Scottish ethnicity) was recently described (Wang et al., 2016).

3.6.6 N-glycosylation in Prognosis

Acute systemic inflammation is a part of many pathological events and a patient's inflammatory response often determines the outcome of a disease. Given that N-glycosylation can meditate the inflammatory effect of IgG, N-glycan biomarkers may be crucial for disease prognosis. One study reported that a rapid increase in galactosylated glycoforms was associated with nearly double the mortality risk measured by EuroSCORE II following cardiac surgery (Novokmet et al., 2014). Another study

reported that serum IgG Fc glycosylation in Guillain-Barré syndrome (GBS) is related to disease severity and clinical recovery after intravenous immunoglobulin therapy and may help to develop new measures to monitor the efficacy of treatment (Fokkink et al., 2014). A separate study reported that treatment response in Kawasaki disease is associated with sialylation levels of endogenous, but not therapeutic, intravenous IgG (Ogata et al., 2013).

3.7 Congenital Disorders of Glycosylation

Although glycosylation is not a direct template driven biosynthetic process, it is regulated by at least 1% of genes, termed "glyco-genes" (Janković, 2011). A high proportion of these genes encode specific enzymes that promote the glycosylation process (Eklund & Freeze, 2006). Alterations, such as a point mutation, can change the orientation of genes at glycosylation sites, thereby preventing the glycosylation of the given polypeptide chains and affecting glycoprotein capabilities (Jaeken & van den Heuvel, 2014). It is, therefore, not a surprise that an alteration in the genetic and the molecular machinery can lead to human diseases, generally termed congenital disorders of glycosylation (CDGs) (Jaeken, 2011; Jaeken & Matthijs, 2007; Rosnoblet et al., 2013).

CDGs are genetic or inherited disorders due to defects in the glycosylation process that form glycoproteins and glycolipids (Grünewald et al., 2002; Heywood et al., 2013). Diverse enzymes, including glycosyltransferases and glycosidases, catalyse the biosynthesis of glycan structures, and mutations in the genes encoding for these enzymes lead to CDGs (Helander, Bergström, & Freeze, 2004). Presently, there are two main types of CDGs. CDG type I arises from mutations that affect the assembly of N-glycans to polypeptide chains in the cytosol of the ER (Cummings & Pierce, 2009). CDG type II arises from mutations that lead to N-glycan processing defects in the Golgi apparatus (Barone, Sturiale, & Garozzo, 2009; Cummings & Pierce, 2009). Other subgroups also exist based on the given gene mutation.

The first and most widely used method is to detect under-glycosylated serum transferrin (Tf) by isoelectric focusing (IEF) electrophoresis (Freeze, 2006; Grünewald et al., 2002). Tf has two N-glycosylation sites with branched glycans attached to Asn 432 and Asn 630 (Freeze, 2006; Guillard et al., 2011). Glycosylation defects modify the pattern of sialylation to these sites, leading to a decrease in tetrasilo-Tf, an increase in disialo or asialo-Tf in CDG type I, and an increase in monosialo and trisialo-Tf in CDG type II (Barone et al., 2009; Guillard et al., 2011; Jaeken, 2011).

Over the years, several analytical methods have emerged for diagnosing and identifying CDGs. The various diagnostic approaches can be classified into glycan structure analysis, enzymatic assays, lipid-linked oligosaccharide analysis, and molecular diagnostics (Grünewald et al., 2002).

Table 3.2 Congenital disorders of glycosylation

CDG-type	Gene	Enzyme	Reaction	Symptoms	Diagnosis
CDG-1a	PMM2	Phosphomannomutase	Man-6-Phosphate ⇅ Man-1-Phosphate	Ataxia, inverted nipples, Psycho-motor retardation (Grünewald et al., 2002)	Transferrin, hypoglycosylation Test, enzymatic assays (Eklund & Freeze, 2006)
		(Hansen, Frank, & Casanova, 1997)			
CDG-Ib	MPI	Phosphomannose isomerase	Fructose-6-P ⇅ Man-6-phosphate	Diarrhoea, vomiting seizures,hypoglycae- mia (Grünewald et al., 2002; Haeuptle & Hennet, 2009)	Enzymatic assays (Eklund & Freeze, 2006)
		(Niehues et al., 1998)		Coaguloparthy, strabism, seizure	Mutational analysis (Eklund & Freeze, 2006)
CDG-Id	ALG3	Mannosyltransferase VI	$Man_5GlcNAc_2$-PP-Dol ⇅ $Man_6GlcNAc_2$-PP-Dol	Dysmorphic features, Cardiorespiratory problems, Hypotonia	Genetic analysis
		(Cummings & Pierce, 2009)			
CDG-Ih	ALG8	Glucosyltransferase 2	$Glc_1Man_9GlcNAc_2$-PP-Dol ↓ $Glc_2Man_9Glc_1NAc_2$-PP-Dol	Psychomotor retardation, hypogonadism (Supraha Goreta, Dabelic, & Dumic, 2012)	Enzymatic assays and genetic analysis (Eklund & Freeze, 2006)
		(Haeuptle & Hennet, 2009)			
CDG-Ik	ALG1	Mannosyltransferase	$GlcNAc_2$-PP-Dol ↓ $Man_1GlcNAc_2$-PP-Dol	Seizures, hypotonia, Dysmorphic features(Haeuptle & Hennet, 2009)	Mutational analysis
		(Cummings & Pierce, 2009)			
CDG-In	RFT1	RFT1 protein	$Man_5GlcNAc_2$-PP-Dol ↓ ER	Sensorineural deafness, cerebellar hypoplasia	Genetic analysis (Eklund & Freeze, 2006)
		(Haeuptle & Hennet, 2009)			
CDG-IL	DIBD1	Glycosyltransferase	$Man_5GlcNAc_2$-PP-Dol ↓ $Man_5GlcNAc_2$-PP-Dol	Muscular hypotonia facial dysmorphism	Enzymatic assays Lipid linked oligosaccharide analysis (Eklund & Freeze, 2006)
		(Haeuptle & Hennet, 2009)			
CDG-IIa	MGAT2	GlcNAc transferase II	GlcNAc-Man3-GlcNAc-Protein ↓ $GlcNAc_2$-Man_3-protein	Seizures, muscular hypotonia, recurrent Edema (Grünewald et al., 2002)	Enzymatic assays
		(Eklund & Freeze, 2006)			
CDG-IIb	GCS1	α-glycosidase	$Glc_3Man_9GlcNA_2$-protein ↓ $Glc_2Man_9GlcNA_2$-protein		
		(De Praeter et al., 2000)			

3.8 Analytical Techniques in Glycomics

Since glycomics became a recognised discipline, developing techniques for complete N-glycan analysis has been challenging, mainly because of the complex nature of N-glycans. In fact, over 200 glycosyltransferases are involved in their biosynthesis, with synthesized forms having different glycosylic linkages, varied anomeric orientations and different degrees of branching (Hizal et al., 2014; Stanley & Cummings, 2009). Consequently, unravelling the structural architecture of these complex biomolecules to appreciate their role in pathophysiology is difficult unless highly sensitive and robust analytical techniques are employed. Generally, N-glycans are analysed by MS (Bindila & Peter-Katalinić, 2009), liquid chromatography (LC) and capillary electrophoresis (CE) (Raman et al., 2005). While this review will not provide a detailed overview of these techniques, a concise introduction on the mode of operation will be necessary.

3.8.1 Mass Spectrometry

Mass spectrometers operate by the formation of charged ions and separate ions according to their mass-to-charge ratio (m/z). Two main ionisation modes are currently known: matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) (Wuhrer, 2013). Briefly, MALDI involves dissolving an analyte in a solution of organic molecules (matrix) which is dried to form solid analyte matrix crystals. Matrix crystals are heated and irradiated by laser pulses causing sublimation into a gaseous phase. Although the exact origin of the ions is not completely clear, it is suggested that ions for MALDI are generated during proton transfer and photon-atomic/molecular interaction (Hoffmann & Stroobant, 2001). Gaseous ions then migrate via an electrostatic field to the analyser. The peculiarity of MALDI is its ability to ionise and desorb analytes in a single run allowing an in depth detection even at the femtomole level (Hoffmann & Stroobant, 2001).

ESI, however, involves the application of a high voltage or electric field to a liquid-analyte mixture via a capillary tube, forming charged droplets. The liquid droplets undergo repeated evaporation as they pass through an inert gas-heated capillary tube. As solvent evaporation is continuous, offspring droplets are formed which increase with increasing electric field intensity, resulting in multiply charged ions that are then analysed. A unique feature of ESI is that the multiply charged ions from large molecular weight molecules are easily detected even on analysers with poor mass limits, making it

very useful for large-scale glycomics (Hoffmann & Stroobant, 2001; Zaia, 2008). Overall, both modes can ionise N-glycans but regardless of which mode is chosen, derivatisation is critical for detection, identification and resolution of the MS spectra (Harvey, 2011; Weiskopf, Vouros, & Harvey, 1998).

A typical example of MS method that has become popular in glycomics is the MALDI-Time of Flight MS (MALDI-TOF-MS). This technique has its strengths and weaknesses. The main advantages include: 1) a low cost per sample because of high-throughput per instrument; 2) the ability to perform site-specific glycosylation analysis; 3) high sensitivity; and 4) adequate structural elucidation. Some of its main disadvantages are: 1) a loss of sialic acid linkages; 2) unable to achieve isomer separation; and 3) it is not reliable for effective quantification (Huffman et al., 2014). Nonetheless, successful application of MALDI-TOF-MS in population-based glycomics is recognised. A typical illustration was when Zuzana and colleagues (2007) examined N-glycan variations in 10 healthy individuals and 24 prostate cancer patients. In brief, serum samples were alkylated and reduced in the presence of ammonium bicarbonate and dithiothreitol (DTT). Aided by PNGase F, serum glycoproteins were deglycosylated and released N-glycans were then extracted by loading onto activated charcoal micro columns in the presence of acetonitrile and trifluroacetic acid (TFA). Eluted N-glycans were derivatised by solid-phase permethylation using NaOH, methyl iodide and dimethylsulfoxide (DMSO). Aliquots of the permethylated N-glycans were then loaded onto a MALDI-plate and at 355 nm wavelength, N-glycan peaks (spectra) of both healthy and prostate cancer patients were analysed (**Figure 3.6**). Employing this technique, the authors identified over 50 N-glycan structures of which 12 structures differed significantly between healthy and prostate cancer patients (Zuzana et al., 2007).

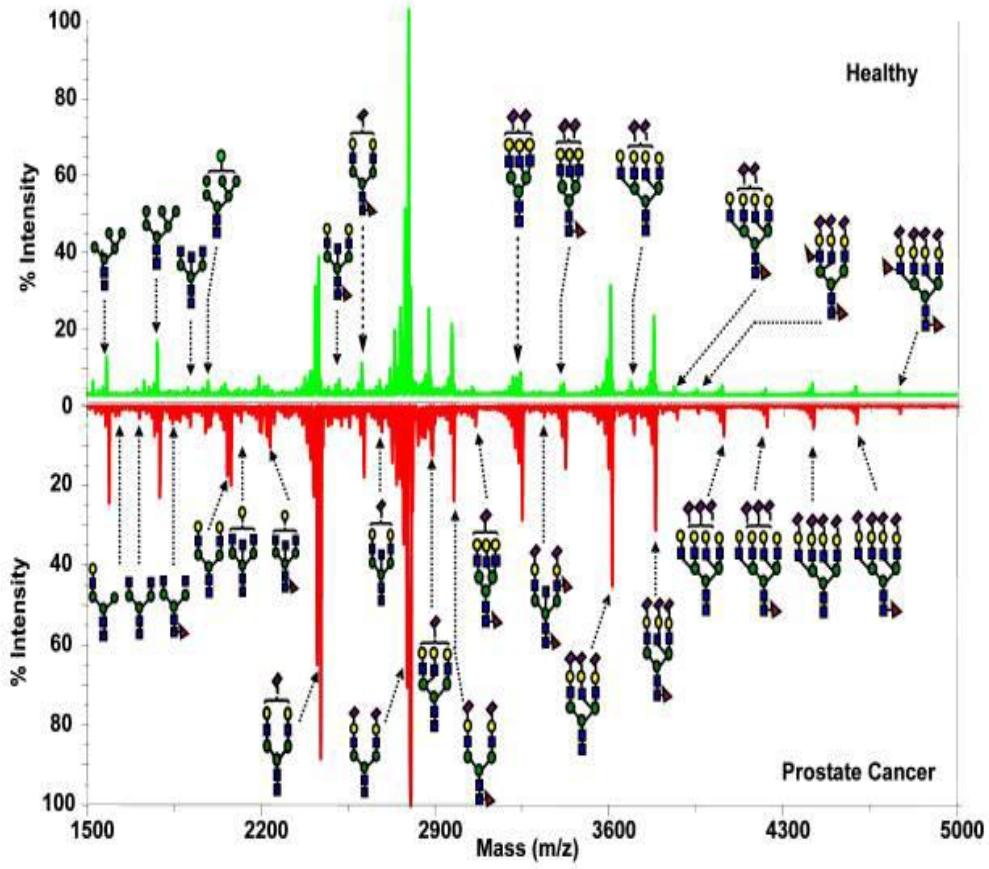

Figure 3.6 MALDI spectra of N-glycans in prostate cancer and controls. MALDI spectra of N-glycans in prostate cancer and controls. Circles, mannose; triangles fucose; squares Nacetylglucosamine; rhomboid N-acetylneuraminic acid. MALDI, matrix-assisted laser desorption–ionisation. Figure reproduced with permission from Zuzana et al., 2007.

In recent years, MALDI-MS Imaging (MALDI-MSI) has been developed to spatially resolve N-glycan structures and provide molecular distribution or maps of the relative amount of each N-glycan trait in a given tissue section (Powers et al., 2013). MALDI-MSI has several advantages: 1) multiple N-glycan structures can be determined in a single run, allowing complete profiling of all analytes present within a tissue; 2) a combination of MSI with other quantitative MS provides an unprecedented profile of tissue specific N-glycan variations (Eshghi et al., 2014); and 3) N-glycans which are visualised in the form of images, allow the sensitive detection of the morphological changes of structures in each tissue section. MALDI-MSI was employed for imaging N-glycans in formalin-fixed paraffin embedded (FFPE) tissues of ovarian cancer patients (Everest-Dass et al., 2016). Shortly thereafter, tissues from ovarian cancer patients were dipped in a formalin solution. After rinsing in deionised water, the tissues were processed in xylene, ethanol and paraffin to form paraffin complexed tissue blocks. Following

further washing with ethanol and incubation with NH_4HCO_3, N-glycans within tissues were released using PNGase F. Released N-glycans were then purified on porous graphitised carbon and cation exchange columns and analysed using porous gel chromatography (PGC)-LC-ESI-MS/MS while MSI was used for visualising the spatial distribution of N-glycans in ovarian cancer tissues (Everest-Dass et al., 2016). It was observed that high mannose and hybrid/complex N-glycans were abundant in the tumour tissues and the stroma respectively (**Figure 3.7**).

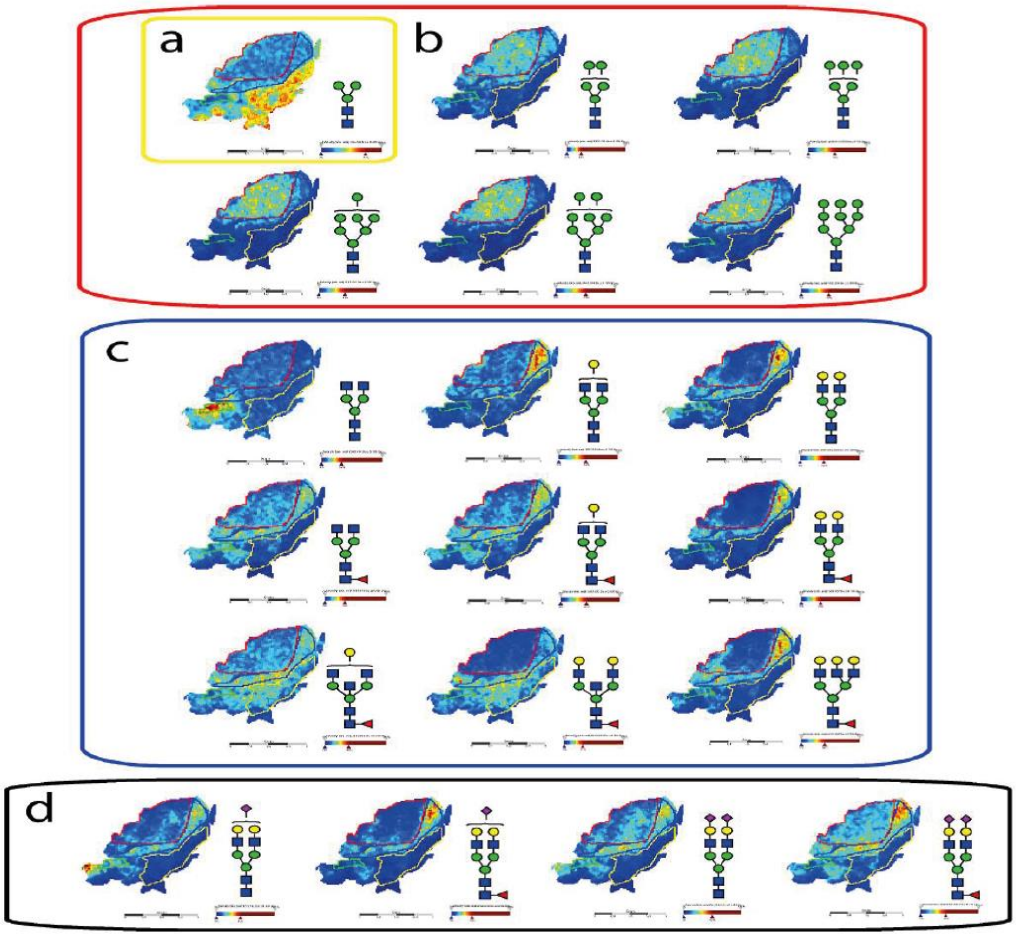

Figure 3.7. N-glycan in formalin-fixed paraffin-embedded tissue sections analysed using MSI. (a) Adipose and necrotic tissues, which predominately contained a pauci mannose structure and an agalactosylated structure (m/z 1339.4. Images in (b) depict tumour areas in the tissues, which predominately contained agalactosylated N-glycans. Images in (c) depict tumour areas in the tissues, which were mostly characterised by high-mannose N-glycans with m/z ranging from 1257.4 to 1905.6. In the stroma (c), were high levels of complex/hybrid N-glycans with m/z ranging from 1501.5 to 2174.7, while sialylated N-glycans were mostly found in (d) with m/z from 1954.6 to 2391.8. MSI, mass spectrometry imaging. Figure reproduced with permission from Everest-Dass et al., 2016.

Apart from MALDI-MSI, other highly sensitive MS equipment are tandem MS, chip-based MS (Bindila & Peter-Katalinić, 2009), Fourier transform ion cyclotron MS (FT-ICR-MS) (Park & Lebrilla, 2005) and surface-enhanced laser desorption/ionisation (SELDI) (Cummings & Pierce, 2009; Lebrecht et al., 2009). While N-glycan analysis using MS has been remarkable, it is limited by its inability to provide detailed information of structural isomers. This has necessitated the coupling of MS to other chromatographic techniques for in-depth structural characterisation (Wuhrer, 2013).

3.8.3 Liquid Chromatography

Rapid advances in technology have made it possible to resolve N-glycan structures using chromatographic techniques. While recognising different separation methods such as gel permeation chromatography (GPC) and graphitised carbon chromatography (GCC), this review will, focus on high-performance liquid chromatography (HPLC) based methods. HPLC separates complex N-glycans by the adsorptive interaction of N-glycans in solution (mobile phase) with a stationary phase. Solvents mostly used as mobile phases include ethanol, methanol and acetonitrile, while amides, silanols/silica gels/particles and diols are often used as stationary phases (Novotny, Alley, & Mann, 2013; Zauner, Deelder, & Wuhrer, 2011). In HPLC, N-glycans in solution are first loaded and after gradually altering the conditions of the mobile phase, N-glycans are eluted based on their retention times on the column, generating peaks (chromatogram) which are observed on a detector (Rudd et al., 2001). Constituent N-glycan monosaccharides are often expressed as glucose units (GU) and peaks are quantified by calculating the area under curve and dividing by the sum of the individual peaks (Royle et al., 2008).

Depending on the composition of the mobile and stationary phases, HPLC can be either a normal phase (NP) or a reverse-phase (RP). While NP-HPLC employs a polar stationary phase and a non-polar mobile phase, RP-HPLC is the direct opposite. Although HPLC has been useful for separating N-glycans, it is limited by possible co-elution and hence, requires several purification steps, which leads to long sample runs and high solvent consumption. For this reason, an automated and highly sensitive form of HPLC known as Hydrophilic Interaction Liquid Chromatography (HILIC) has been developed (Zauner et al., 2011). As an advanced form of HPLC, HILIC employs a partition mechanism to separate N-glycans according to their charge, size and oligosaccharide composition. Very often, N-glycans for HILIC separation are derivitised with 2-AB to enhance UV detection. Since these labels confer some hydrophobic properties, derivitised

N-glycans have weak interaction with the stationary phase, causing them to be eluted first (Zauner et al., 2011). Structural assignment of the eluted N-glycans, expressed as GU are then performed by comparing to reference databases which are discussed later in this review (Royle et al., 2008). HILIC has several advantages over HPLC. Some of these are; a single and efficient separation of both charged (sialylated) and uncharged/ highly polar (OH) N-glycans, full isometric separation and high-resolution potential (Novotny et al., 2013).

In recent years, HPLC-HILIC has been applied to identifying biomarkers for chronic diseases. For example, Lu et al., (2011) investigated the correlation between plasma N-glycan profiles and metabolic syndrome (MetS) among 212 Chinese Han and 520 Croatian individuals. Briefly, N-glycans were released from 5 µl of plasma in a microplate using PNGase F and fluorescently labelled with 2-AB. This was followed by exoglycosidase digestion with *Arthrobacter ureafaciens* sialidase. Using a TSK gel Amide-80 5-µm (250 x 4.6 mm) column, formic acid and ammonia buffer, with acetonitrile as the organic solvent, N-glycans were separated on HPLC-HILIC. Weak anion exchange (WAX)-HPLC was used to separate complex N-glycans based on the number of sialic acid groups attached. Using this technique, it was possible to assign 16 N-glycan peaks (**Figure 3.8**) of which 9 significantly correlated with established MetS biomarkers including systolic blood pressure, diastolic blood pressure, body mass index and fasting blood sugar (Lu et al., 2011).Similarly, McLachlan et al., (2016) employed the HPLC-HILIC technique to investigate the association between plasma N-glycans and MetS in a large Orcadian population comprising 2,039 individuals. This study identified 21 N-glycan traits that were altered in MetS (McLachlan et al., 2016).

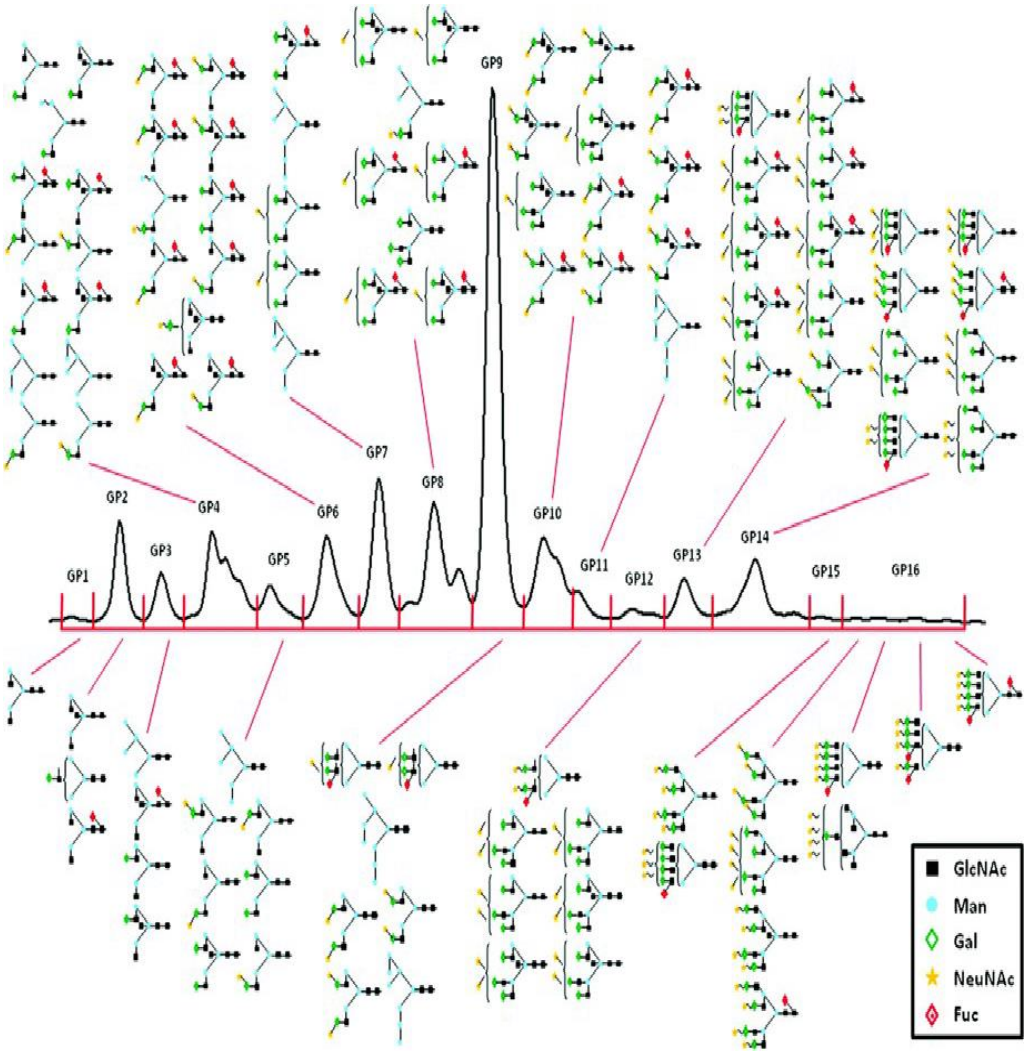

Figure 3.8. Plasma N-glycome Enabled by HPLC-HILIC. Thirty-three plasma N-glycan peaks from MetS in Chinese Han and Croatian populations were categorised into 16 groups. Structural assignment of each N-glycan peak was determined after comparing with a reference database. HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; MetS, metabolic syndrome. Figure reproduced with permission from Lu et al., 2011.

Progressing from HPLC, ultra-performance liquid chromatography (UPLC) has been designed, allowing shorter analytic runs, less solvent consumption and improved resolution (Saldova et al., 2014). Like HPLC-HILIC, UPLC has been employed for studying N-glycan structures on a large scale **(Figure 3.9)**. An example of such a study is by Saldova et al., (2014), who examined N-glycan profiles in the serum of 62 breast cancer patients and 107 healthy controls. Briefly, N-glycans were released from the serum using PNGase F after which they were fluorescently labelled with 2-AB. Following several washing steps, N-glycans were separated on UPLC using 1.7 BEH Glycan in a 2.1 × 150 mm column. WAX-HPLC was then performed to separate the N-glycans based

on their sialic acid attachments and confirmed by exoglycosidase digestions. Through this technique, over 140 N-glycans were assigned which are potential biomarkers for breast cancer (Saldova et al., 2014).

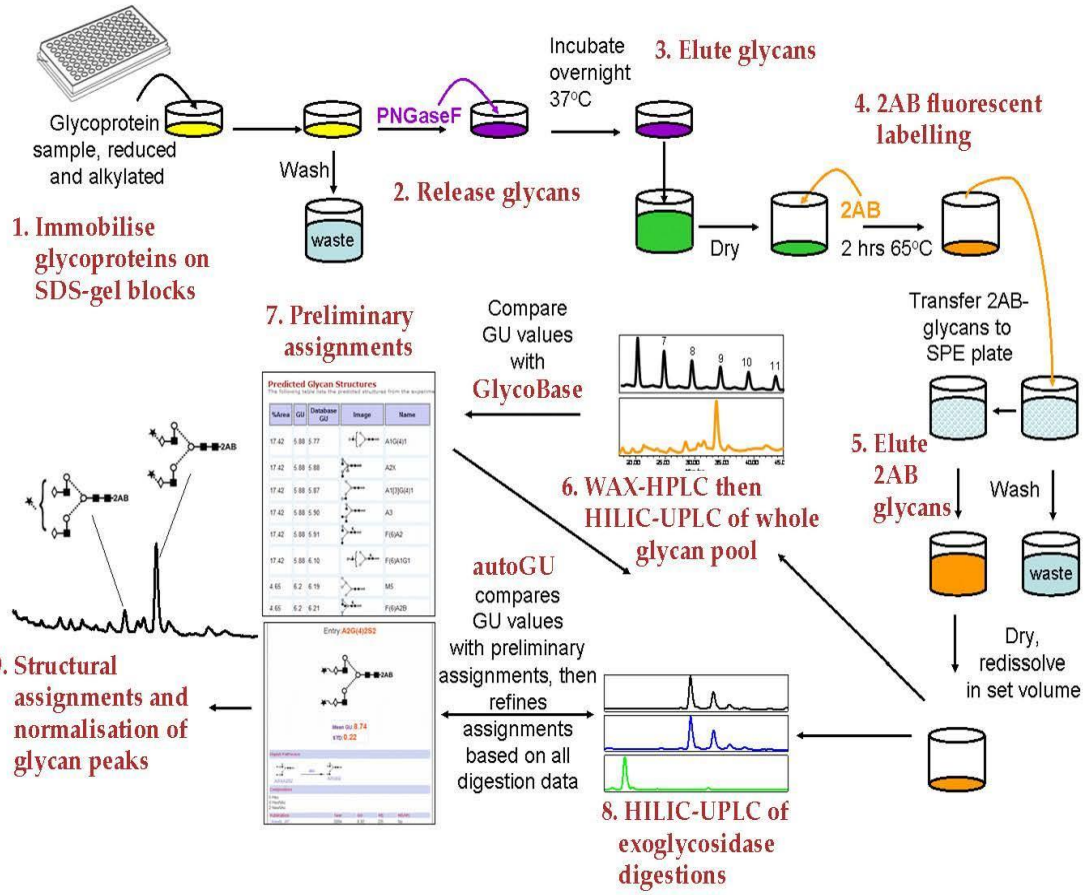

Figure 3.9 N-glycosylation analysis by UPLC-HILIC

Glycoproteins are immobilised on 96 well plates, denatured with sodium dodecyl sulfate (SDS), and washed. N-glycans are freed with PNGase F and incubated at 37°C. After elution, N-glycans are labelled with 2-aminobenzamine (2-AB) and followed by a series of washing and elution steps. N-glycans are separated by hydrophilic interaction chromatography on a Waters Acquity ultra performance liquid chromatography (UPLC) instrument (Waters Corporation, Milford, MA, USA). Structural assignments are performed by reference database matching. Figure is modified from Doherty et al., 2012.

Similarly, Krištić et al., (2014) used UPLC to analyse immunoglobin G (IgG) in the plasma of 5,117 individuals from four European populations and observed major IgG changes such as fucosylation, bisecting G1NAc and sialylation that correlated with age. In particular, after analysing 24 IgG glycan peaks from the chromatogram (**Figure 3.10**), it was concluded that nongalactosylation was positively associated with age while digalactosylation decreased with age. These findings therefore show that glycosylation changes in IgG can be an important predictor of human aging (Krištić et al., 2014). Other scientists who have employed the UPLC technique for N-glycan analysis include Yu et

al., 2016; Wang et al., 2016 and Sebastian et al., 2016). Although not presently well known, another innovative technique that will soon flood the glycomic field is UPLC-MS (e.g. UPLC-QTof). This technique allows the detection of multiple features in a given sample and exploits the advantages of MS for a better MS/MS glycan fragmentation and perfect mass confirmation of low-level peaks (Dunn et al., 2011).

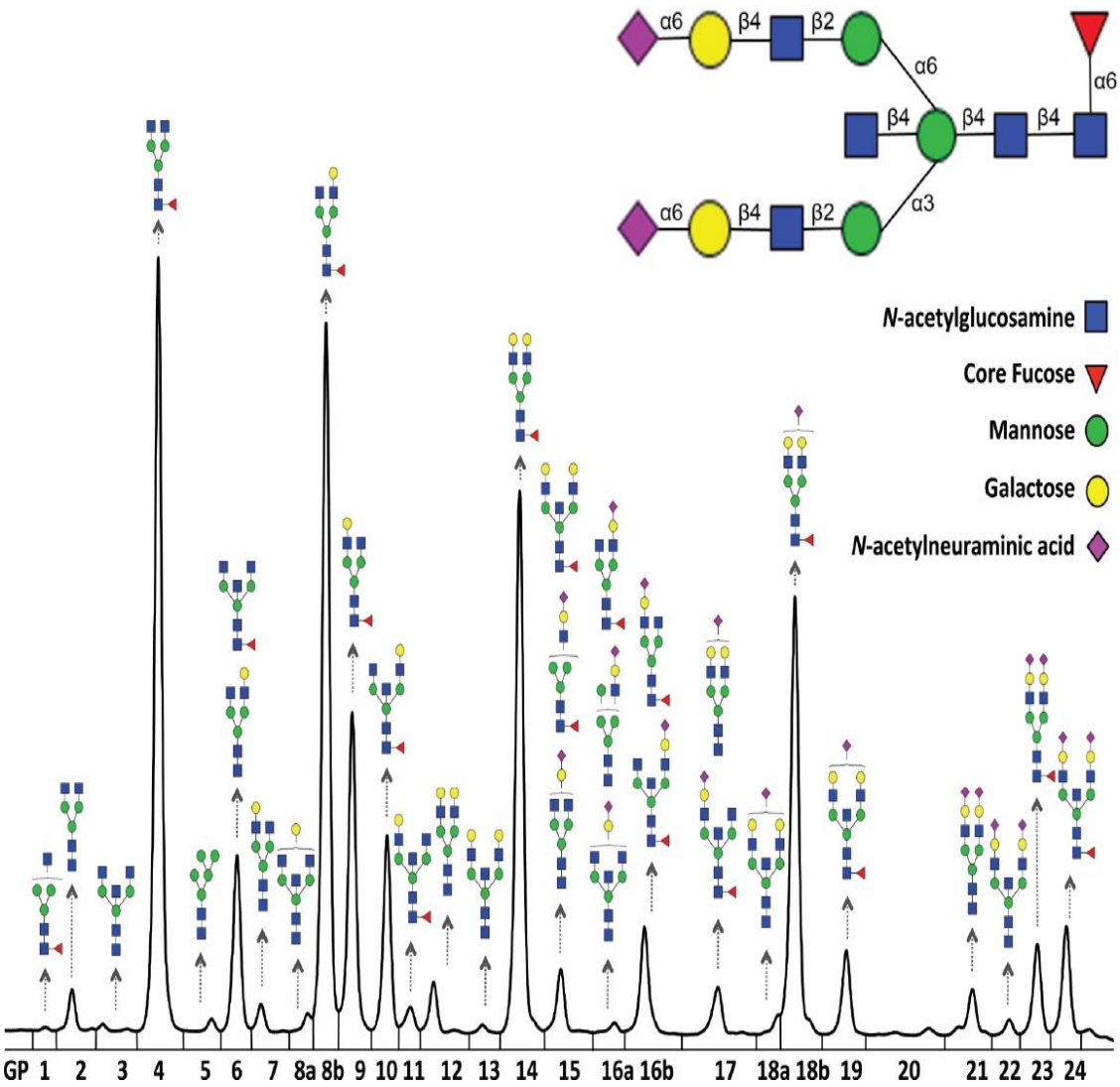

Figure 3.10. IgG glycosylation profiles using UPLC. Twenty-four N-glycan peaks were generated on the UPLC chromatogram. UPLC, ultra-performance liquid chromatography. Figure reproduced from Kristic´ et al. 2014.

3.8.3 Capillary Electrophoresis

Capillary electrophoresis (CE), since its emergence in the past few years, has been considered one of the most robust techniques for analysing N-glycans (Mechref, 2011; Rowena & Terabe, 2005). In principle, CE separates charged ions in solution in a narrow separating capillary tube. This capillary tube is dipped in two buffer-containing reservoirs connected to a high voltage source. When the analyte is introduced at the inlet of the reservoir, a voltage is applied generating an electric field, which causes the analyte to migrate. Based on their mass-to-charge ratios, the analytes are distinctly separated on the detector while generating an electropherogram on the data acquisition device (Altria, 1996; Ewing, 1989).

CE instrumentation is simple yet capable of distinguishing structural isomers which otherwise are not easily separated using MS (Mechref, 2011). Additionally, it is fast, efficient and requires only a small sample volume for analysis, making it an effective tool in situations where there is limited sample quantity (Zhao et al., 2012). In contrast to other analytical techniques, CE separation can be achieved under mild conditions, with low salt concentrations thereby protecting the integrity of the analyte (Rowena & Terabe, 2005). However, comprehensive and detailed information of the N-glycan structure can be obtained when CE is coupled to other analytical techniques such as laser-induced florescence (LIF). Similar to the other described techniques, CE-LIF will require derivatisation with fluorescent tags for sensitive detection. Among the most commonly, used tags are *p*-amino benzoic acid (*p*-ABA), 7-amino-1, 3-naphthalene disulfonic acid (ANDSA) and 2-aminonaphthalene-1-sulfonic acid (ANS). The main drawback of CE is that it is unable to perform site-specific glycosylation analysis (Huffman et al., 2014).

Varadl et al., 2013 who profiled N-glycans of haptoglobin in pneumonia, chronic obstructive pulmonary disease (COPD) and lung cancer, described a clinical application of this technique. Briefly, plasma samples were washed with a binding buffer and after incubation; samples were centrifuged to separate the bound haptoglobin from the unbound material. The bound haptoglobin was then loaded onto a column containing a specific haptoglobin monoclonal antibody. Following several washing steps with phosphate buffered saline (PBS), haptoglobin was eluted and purified. N-glycans in haptoglobin were released using PNGase F and fluorescently labelled with APTS. Sialic acids on the complex N-glycans were then digested using exoglycosidase sialidase and the resultant profiles analysed on CE-LIF (**Figure 3.11**). As shown by this technique, there was an increase in α-1, 6-triantennary glycans in all patient groups compared to

controls. Additionally, when compared to COPD, lung cancer patients showed increased core and arm fucosylated tetraantennary N-glycans (Varadl, 2013).

Similarly, by applying the CE-LIF technique, Schwedler and colleagues (2014) were able to identify 34 and 32 N-glycan isomers in the serum of epithelial ovarian cancer (EOC) patients and healthy patients respectively. They found that compared to healthy patients, core fucosylated tetraantennary N-glycans were higher in EOC, while diantennary and high mannose N-glycans decreased (Schwedler, 2014).

Other types of CE-based techniques often employed for N-glycan analysis are capillary zone electrophoresis (CZE), and micellar electrokinetic chromatography (MEKC).

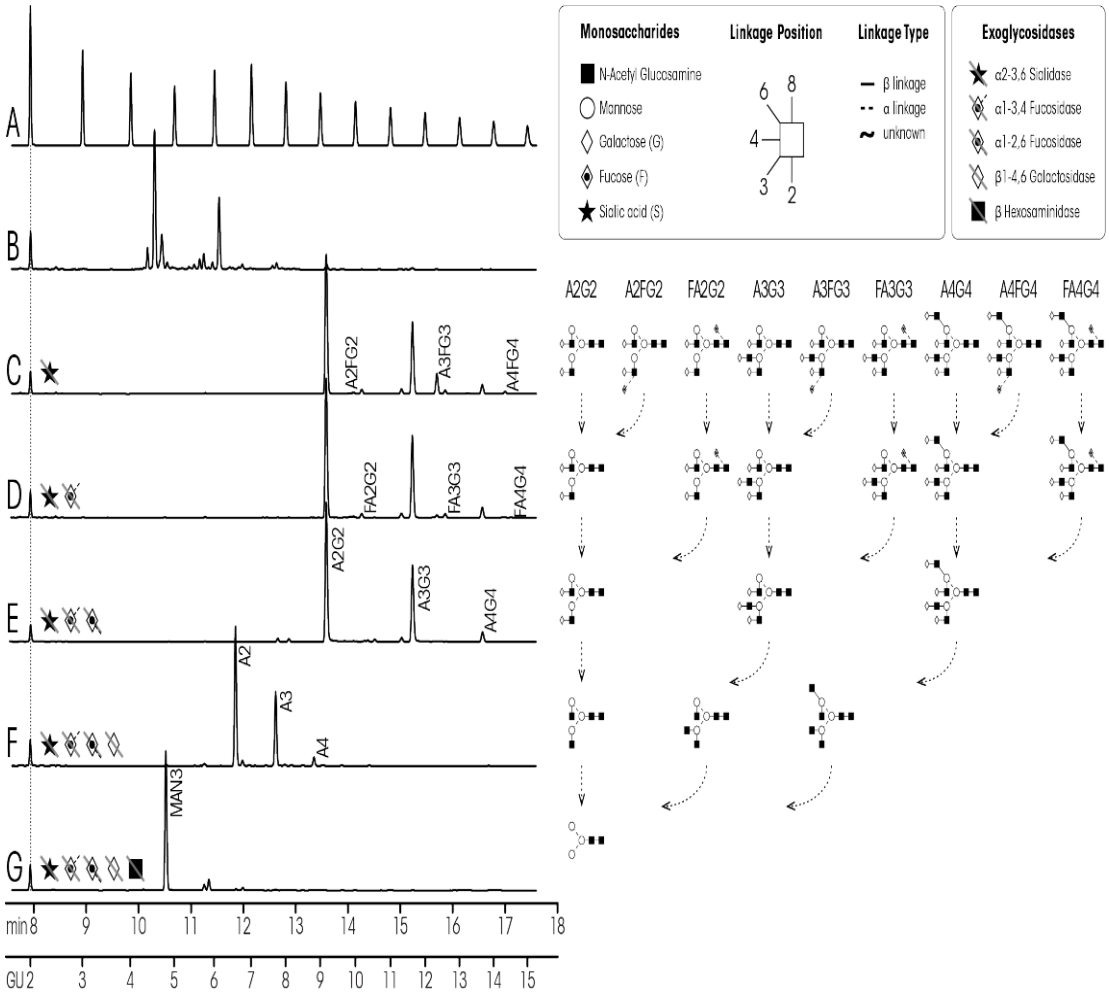

Figure 3.11 N-glycan profiles of plasma haptoglobin in pneumonia, lung cancer, COPD, and controls. Different exoglycosidases such as sialidase, fucosidase, hexosaminidase, and galactosidase were used to digest haptoglobin and profiled using CE-LIF. CE, capillary electrophoresis; COPD, chronic obstructive pulmonary disease. Figure reproduced with permission from Varadl et al., (2013).

Despite the advancement of analytical and deglycosylation techniques, the relative complexities of glycan structures cannot be fully elucidated with these techniques alone. Adequate and comprehensive N-glycan databases are needed to aid experimental data storage, N-glycan structure annotation and interpretation into computer-readable formats that can be easily accessed by glycobiologists.

3.9 Glycoinformatics Tools

When compared to genomics and proteomics where there are large pools of databases, comparable databases for glycomics are still in the early stages (Hayes et al., 2011; Krishnamoorthy & Mahal, 2009; Lazar et al., 2013; Raman et al., 2005; Von Der Lieth et al., 2004; Turnbull & Field, 2007). Notwithstanding this, a large pool of glycoinformatic databases containing a repertoire of N-glycan structures have been developed (Artemenko, Campbell, & Rudd, 2010; Campbell et al., 2008; Hayes et al., 2011; Von Der Lieth et al., 2011). Many of these databases are not well known due to inadequate resources, website inaccessibility and lack of public awareness (Hizal et al., 2014). While this review will not fully cover all the N-glycan databases known, it will highlight some of them.

The first attempt to set up glycan databases was in the early 1990s when the Complex Carbohydrate Structure Database (CCSD), often called the CarbBank, was established by the Complex Carbohydrate Research Centre at the University of Georgia (Doubet & Albersheim, 1992; Frank & Schloissnig, 2010; Lütteke et al., 2006). They created a catalogue of 9,200 carbohydrate sequences, which were pooled from over 2500 publications during the early-1990s (Doubet & Albersheim, 1992). Lack of funding unfortunately led to this group discontinuing their efforts; however, their pioneering work led to the development of all the modern databases for glycomics research (Frank & Schloissnig, 2010; Von Der Lieth et al., 2011).

3.9.1 EUROCarbDB

The EUROCarbDB is a web-based, open access resource, which contains a cluster of N-glycan profile data from the HPLC and MS platforms (Campbell et al., 2008; Frank & Schloissnig, 2010). It also has unique features for storing curated and experimental data, and tools for visualising N-glycan structures (Von der Lieth et al., 2011). For example, it is possible to completely observe N-glycan structures, including their anomeric orientation, stereoisomers, and the type of monosaccharide linkages because of the embedded GlycanBuilder tool designed for an intuitive graphical visualisation

(Ceroni, 2007; Von Der Lieth et al., 2011). Importantly, it is possible to incorporate and integrate other databases in the EUROCarbDB platform to promote data exchange and accessibility. Given the structural diversity of N-glycans, uniform annotation of monosaccharides is challenging. However, in EUROCarbDB there is an embedded GlycoCT and MonosaccharideDB, which uses a controlled vocabulary for annotating constituent monosaccharides with unified names that are easily translated to computer-readable formats (Ceroni, 2007; Aoki-Kinoshita, 2008; Frank & Schloissnig, 2010).

3.9.2 GlycoBase

GlycoBase is an open access resource containing N-glycan data for classifying and assigning N-glycan structures from the HPLC, UPLC and CE platforms (Hizal et al., 2014; Saldova et al., 2014; Stöckmann et al., 2013). It is a repository of over 350 2-AB labelled N-linked structures in GU, and provides information of N-glycan positions and their exoglycosidase digest (Campbell et al., 2008). Likewise, with GlycoBase, it is also possible to visualise constituent monosaccharides and their linkage orientation (Campbell et al., 2008). Advancing GlycoBase is the data matching automation software, autoGU that assigns N-glycan peaks from HPLC and provides information of their exoglycosidase products (Artemenko et al., 2010; Campbell et al., 2008). While the GlycoBase software has improved glycomics research, the cumbersome task of manually exporting sample sets to other file formats and the likelihood of data integration errors limits its use (Artemenko et al., 2010). This appears to be overcome by the GlycoExtractor database (Artemenko et al., 2010).

3.9.3 GlycoExtractor

GlycoExtractor is another web-based resource for analysing spectra from HPLC, HILIC and MS platforms (Artemenko et al., 2010; Saldova et al., 2011; Saldova et al., 2014). In GlycoExtractor, samples are assigned based on their peak number and the experimental date, and exports desired file formats to other platforms such as EUROCarbDB for detailed structural assignments (Artemenko et al., 2010; Hizal et al., 2014). Unlike the GlycoBase, data extraction and exporting in GlycoExtractor is automated. For example, Artemenko et al., has shown that exporting data from 100 profiles into XML or JSON formats, which manually would have taken 90 minutes to perform, could be done in 1-2 seconds using GlycoExtractor (Artemenko et al., 2010).

3.9.4 GlycomeDB

GlycomeDB is a web-based resource containing a collection of N-glycan structures from several databases and comprises over 35,056 structures (Ranzinger et al., 2009). Embedded GlycoCT and the glycoUpdateDB interfaces makes cross-linking between databases possible, thereby providing complete specific information of each N-glycan (Hizal et al., 2014). In this context, GlycoUpdateDB uploads datasets from multiple databases, translates them into a GlycoCT format and then incorporates them into the GlycomeDB (Hizal et al., 2014). Additionally, species-specific N-glycan data and anomeric configuration of monosaccharides, whether alpha or beta can be obtained using this platform (Ranzinger et al., 2009).

3.9.5 Consortium for Functional Glycomics Database

The Consortium for Functional Glycomics (CFG) database is a large resource containing a collection of glycomics datasets (8,626 glycan sequences) from MALDI-TOF MS, nuclear magnetic resonance (NMR) and N-glycan arrays (Frank & Schloissnig, 2010; Hizal et al., 2014; Von Der Lieth et al., 2011). In the CFG, it is possible to search for an array of N-glycan profiles because it integrates other O- and N-databases such as CarbBank and glycominds. Here, N-glycans can be searched by name, molecular mass or monosaccharide composition, and aided by the substructure interphase, researchers are able to build and modify specific N-glycans by comparing with a given template (Hizal et al., 2014; Raman et al., 2006). Enabled by the cartoonist programme, N-glycan structures can be assigned and viewed in the form of a PDF or JPG. In the CFG, data analysis is hierarchical. For example, N-glycan profiles from tissues and cells using MS are organised according to these hierarchical levels: “species→tissue→sample→N-/O-linked glycan profile→ high/low molecular weight glycans” (Raman et al., 2006).

3.9.6 GLYCOSCIENCE.de

The GLYCOSCIENCE.de is a web-based resource containing N-glycan structural data from NMR and MS platforms, and comprises over 23,233 structures. GLYCOSCIENCE.de is able to link different data sources, such as the protein data bank (PDB) and the CCSD, for adequate structural description (Frank & Schloissnig, 2010; Lütteke et al., 2006; Von Der Lieth et al., 2004). The complex nature of the glycans requires a unique encoding scheme that describes all the chains in the glycan structure. GLYCOSCIENCE.de applies the LInear Notation for Unique description of Carbohydrate Sequences (LINUCS) scheme that uniquely defines all monosaccharide

linkages present, thus simplifying the glycan search facility within this resource (Lütteke et al., 2006). A very important feature of GLYCOSCIENCE.de is the GlycoCT, which is useful for classifying cell surface macromolecules. Embedded in GLYCOSCIENCE.de are tools that display the three-dimensional representation of the glycan structural coordinates and glycosidic linkages (Aoki-Kinoshita, 2013).

3.9.7 GlycoWorkbench

The GlycoWorkbench is a unique resource designed as part of the EUROCarbDB, which provides a graphical interface for interpreting N-glycan data obtained from MS spectra (Ceroni et al., 2008; Von Der Lieth et al., 2011). In the GlycoWorkBench, N-glycans from experimental MS peaks are first paired with N-glycans in the theoretical list using an *in silico* fragmentation engine. Specified N-glycans annotation in the GlycoWorkBench is achieved by cross-linking with other glycan databases such as the GLYCOSCIENCE.de, the CarbBank and the CFG databases (Ceroni, 2007). The embedded GlycanBuilder allows complete editing and visualisation of glycan structures. In the GlycanBuilder, various symbols can be assigned to monosaccharide units and modification of the glycan structures is possible. For example, the ring size, the anomeric configuration and the linkage types of the glycan structures can be defined (Ceroni et al., 2008).

3.10.1 Conclusion

Glycans are very important biomolecules involved in cellular processes and characterised by complex biosynthetic mechanisms. N-glycan biomarkers are a dynamic tool for understanding the complexities underpinning various pathological conditions that in turn will stimulate therapeutic interventions for diseases. High-throughput analytical techniques have allowed the underlying structure and intricacies of these unique molecules to be unraveled.

However, as interesting as N-glycan profiling is, it is not devoid of challenges. Most of the analytical methods highlighted here are unable to detect the concentration of glycans on a microscale level (Wang, 2013). Some of the methods also require high purity samples, which are difficult to generate. Heterogeneity and the complexity of the glycan structures make N-glycan analysis difficult, warranting the need for new streamlined and automated glycobioinformatic resources (Brooks, 2009). Additionally, only a few laboratories with advanced tools and technical expertise are able to analyse specific glycan structures at glycosylation sites, posing a challenge for glycobiologists who are

new to the field (Cummings & Pierce, 2014).

The field of glycomics is undergoing a revolution and advances in this area are attributed to recent technological innovations that aid N-glycan analysis. When compared to genomics, metabolomics and proteomics where there are well-established databases, glycomics databases are still underdeveloped mainly because of N-glycan heterogeneity and different degrees of branching. Nonetheless, a combination of the few glycomics databases with the highly sensitive analytical techniques have made it possible to understand how aberrant N-glycans are linked to multiple chronic diseases. This suggests that N-glycan profiles might, in the near future, be one of the most robust biomarkers for risk stratification that will improve detection and set the pace for preventive treatments for chronic diseases. Although at present, glycan studies are mainly focused on chronic diseases, it will be intriguing to explore and identify specific N-glycan traits that relate to certain acute diseases.

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CHAPTER FOUR

Prelude

The contextual literature search thus far has shown that the process of developing T2DM may take many decades and therefore the approach for testing the current status of T2DM is improvident. There is the need to recognise early signs of risk to enable an early intervention and delay the onset of the disease. In this present study **(Study I)**, the SHSQ-25 is employed to identify individuals who may have SHS, the reversible stage of a chronic disease like T2DM. Further, this study presents an overview of the health status of participants (T2DM and healthy controls) involved in the overall project. This study has been published in the *Journal of European Association of Predictive, Preventive and Personalised Medicine (EPMA Journal)* and it can be cited as:

Adua, E., Roberts, P. & Wang, W. (2017). Incorporation of suboptimal health status as a potential risk assessment for type II diabetes mellitus: a case-control study in a Ghanaian population. *EPMA Journal*, 8(4), 345-355.

Incorporation of suboptimal health status as a potential risk assessment for type II diabetes mellitus: A case-control study in Ghana

4.1 Abstract

Due to a paradigm shift in lifestyles, there is growing concern that Type 2 Diabetes Mellitus (T2DM) will reach epidemic proportions in Ghana. However, specific characteristics of the disease are under explored in this region. More challenging are those who are yet to be diagnosed or who complain of poor health in the absence of a diagnosed disease - suboptimal health status (SHS). We conducted a study to examine various factors that characterise SHS and T2DM. Using a cross-sectional design, we recruited 264 people as controls and 241 T2DM patients from January to June 2016. The controls were categorised into high and low SHS based on how they rated on the SHS questionnaire-25 (SHSQ-25). Anthropometric and biochemical parameters: body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP), fasting plasma glucose (FPG), glycated haemoglobin (HbA1c), serum lipids [(total cholesterol, triglycerides (TG), high and low-density lipoprotein-cholesterol (HDL-c & LDL-c)] were measured. The male to female ratio for T2DM and controls were 99/142 and 98/166 respectively, while the mean ages were 55.89 and 51.52 years. Compared to controls, T2DM patients had higher FPG (8.96 ± 4.18 vs 6.08 ± 1.79; p<0.0001) and HbA1c (8.23 ± 2.09 vs 5.45 ± 1.00; p<0.0001). Primarily sedentary [aOR=2.97(1.38-6.39); p=0.034)], SBP (p=0.001) and DBP (p=0.001) significantly correlated with high SHS. After adjusting for age and gender, central adiposity [aOR=1.74(1.06-2.83); p=0.027)], underweight [aOR=5.82(1.23-27.52); p=0.018)], high SBP [aOR=1.86(1.14-3.05); p=0.012)], high DBP [aOR=2.39 (1.40-4.07); p=0.001)] and high TG [aOR=2.17(1.09-4.33); p=0.029)] were found to be independent risk factors associated with high SHS. The management of T2DM in Ghana is suboptimal and undiagnosed risk factors remain prevalent. The SHSQ-25 can be translated and applied as a practical tool to screen at-risk individuals and hence prove useful for the purpose of predictive, preventive and personalised medicine.

Key words: chronic diseases, biomarkers, predictive preventive and personalised medicine

4.2 Introduction

The rising prevalence of diabetes mellitus (DM) is a major health threat worldwide. Presently, DM affects more than 422 million people with an enormous proportion (≈90%) of these being type II diabetes mellitus (T2DM) (WHO, 2015). Data from the World Health Organisation (WHO, 2015) and the International Diabetes Federation (IDF) (IDF, 2015) suggests that T2DM is directly related to urbanisation, mechanisation, physical inactivity and unhealthy diet and since many adults are still adopting these characteristics, the prevalence of T2DM is likely to escalate. The projected trajectory of prevalence in the years 2025-2030 is 500 million worldwide (Golubnitschaja, Kinkorova, & Costigliola, 2014). More disconcerting is the increasing prevalence of the disease among adolescents and young adults. These individuals are likely to spend more on medical costs and have more time to suffer from both microvascular and macrovascular complications than older adults (Bao et al., 2017; Chew, 2017; Nadeau et al., 2016).

T2DM affects multiple organs in the human body and people with the disease have an elevated risk of blindness, cognitive decline, kidney failure, cardiovascular diseases, fractures, brain damage, depression and consequently premature death (Action to Control Cardiovascular Risk in Diabetes Study Group, 2008; Chen, Magliano, & Zimmet, 2012; Gerstein et al., 2005; Luchsinger, 2012; Rich, Shaefer, Parkin, & Edelman, 2013; Stratton et al., 2000). Many of these complications may be averted or delayed with timely health education and intervention (Adua et al., 2017; DeFronzo & Abdul-Ghani, 2011; Frank et al., 2014; Rich et al., 2013; Suckling & Swift, 2015). Unfortunately, the majority of people, especially those residing in less healthcare-resourced and low income developing countries are not aware of their risk status.

In Ghana, up to 440, 000 people had been documented to have T2DM in 2013 but the number of those with pre-diabetes are not known (Guariguata et al., 2014). These individuals can remain undiagnosed for a long period, even for many decades of their life. Effective therapeutic intervention can only be started following the manifestation of clinical symptoms. This, from the perspective of a preventive, predictive and personalised medicine (PPPM) standpoint is a delayed response (Golubnitschaja et al., 2014). PPPM is defined as "an integrative concept that enables the prediction of individual's predisposition before the onset of a disease, to provide targeted preventive measures and create personalised treatment algorithms tailored to a person" (Lemke & Golubnitschaja, 2014). Over the past few years, PPPM has made a significant impact on the prevention

and treatment of diseases because it adopts a holistic approach (e.g. environmental, behavioural and traditional factors) to solving health problems (Golubnitschaja, 2010; Golubnitschaja et al., 2016; Golubnitschaja et al., 2014).

As with many chronic diseases, screening for pre-diabetes is central in PPPM and it provides the stimulus for initiating treatment and delaying long-term complications. Most often, screening is performed in a health care facility in order to allow health care providers to perform appropriate follow up testing and institute quality health care (Lindström & Tuomilehto, 2003). However, with recent developments in public health research, there are robust screening tools that are non-invasive, inexpensive and can be applied both in a health care setting and in the field or the wider community. One such tool is the suboptimal health status questionnaire (SHSQ-25) (Wang et al., 2016; Yan et al., 2014).

SHSQ-25 identifies individuals who complain of poor health in the absence of any diagnosable condition (Yan et al., 2009). It explores human health from five domains: fatigue, cardiovascular, immune, digestive and mental and over the years, it has been successfully applied for screening purposes among Caucasians (Kupaev et al., 2016) and Chinese (Wang, Russell, & Yan, 2014; Wang et al., 2016; Yan et al., 2009; Yan et al., 2012; Yan et al., 2014). In these studies, it was apparent that a high SHS (i.e. SHS score > median score), is associated with chronic disease risk factors and that these scores are largely under the influence of external factors such as employment type, lifestyle, socioeconomic, cultural and climatic conditions.

In this study, SHS in a Ghanaian population is examined as well as examining the anthropometric, clinical and biochemical parameters among T2DM patients. Understanding these factors in both healthy and T2DM participants will be instrumental in the pursuit of PPPM.

4.3 Methods and Study Design

Data Collection

This cross-sectional study was conducted from January to June 2016. Recruitment for the study was based on purposeful sampling where T2DM patients, who reported at the Diabetic Centre, Komfo Anokye Teaching Hospital (KATH), were invited to participate. KATH is a referral hospital with over 1200 beds with not less than 100 diabetic/hypertensive patients attending the hospital every fortnight. Utilising a

convenient sampling method, 264 control participants from three suburbs (Ash-town, Pankrono and Abrepo) were recruited within the Kumasi metropolis.

Ethics Clearance

The Committee on Human Research, Publication and Ethics (CHRPE), Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, and the Human Research Ethics Committee (HREC), Edith Cowan University (ECU), Australia, reviewed and approved the study protocol. Written informed consent was obtained from all participants. Data was collected in accordance with the principles of the Declaration of Helsinki.

SHSQ-25 Questionnaire

The SHSQ-25 questionnaire was used to measure SHS. The SHSQ-25 comprises 25 items, categorised into five domains: fatigue (9 items), cardiovascular system (3 items), digestive system (3 items), immune system (3 items) and mental health (7 items). Each participant was asked to rate a statement on a five-point Likert type scale, based on how often they had experienced a particular complaint in the previous 3 months. 1) Never or almost never, 2) Occasionally, 3) Often, (4) Very often and (5) Always. The raw scores of 1 to 5 on the SHSQ-25 were recoded as 0-4. SHS score was calculated by summing the ratings for the 25 items. A high SHS score represents poor health (Wang et al., 2014; Wang et al., 2016; Yan et al., 2009; Yan et al., 2014). To test for reliability of the SHSQ-25, we determined the Cronbach's α coefficient that was found to be 0.91.

Anthropometric Examination

Weight (kg) and height (cm) were measured with a standard stadiometer (SECA, Hamburg, Germany). These data were used to determine the body mass index (BMI), calculated as BMI = weight (kg)/height $(m)^2$. Waist and hip circumference were measured in cm using a tape measure and waist-to-hip ratio (WHR) was calculated as WHR = waist (cm)/hip (cm). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using a standard sphygmomanometer (Omron HEM711DLX, UK).

Clinical Data

After an overnight fast, blood samples were collected from each participant. Samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant, gel separator and fluoride oxalate. Samples were centrifuged (Mendelssohn, USA) at 3000g at 4^{o}C for 10 mins (centrifuge Eppendorf 5702R, Germany) to separate the whole blood. Serum levels of total cholesterol (TC),

triglycerides (TG) and high-density lipoprotein (HDL) cholesterol were determined enzymatically with commercial reagents (Elitech Clinical Systems Elitech Group; Roche Diagnostics, COBAS INTEGRA 400 Plus, USA). Serum lipid levels were quantified based on the National Cholesterol Education Program, Adult Treatment Panel (NCEP-ATP) III guidelines. Low-density lipoprotein (LDL) cholesterol was calculated using Friedewald formula: LDL=TC−[HDL+TG/5] (Friedewald, Levy & Fredrickson 1972).

Inclusion and Exclusion Criteria

This study was conducted in consultation with clinicians and qualified health professionals. In this study, T2DM was established based on the international classification of disease (ICD-10-CM Diagnosis Code E11.9). Each patient was carefully examined and his or her medical records thoroughly reviewed. As a result, we excluded all those individuals who were suffering from cancer, arthritis, infectious diseases, cardiovascular disease, thyroid disorders, pituitary disorders and adrenal disorders. The study did not include pregnant and lactating mothers. Since T2DM is largely a disease of ageing, the study recruited only individuals who were 30 years and above. Further, to limit potential confounding and the likelihood of recruiting participants with type 1 diabetes, we excluded participants on insulin injections. In order to screen for individuals with undiagnosed risk factors (controls), we excluded all participants who had been previously diagnosed with diabetes and/or hypertension. In addition, individuals who were suffering from other chronic diseases related to the genitourinary, digestive, respiratory and haematological systems were excluded. We included participants aged 18-80 years.

4.4 Statistical Analysis

All continuous data were recorded as mean ± standard deviation (SD) and percentages for categorical variables. Between group comparisons for continuous variables were determined using student *t*-tests, while intergroup comparisons of categorical variables were done with chi-square tests and analysis of variance (ANOVA). Association between SHS and cardiovascular risk factors were performed using linear regression and multiple logistic regression models. Odds ratios (ORs) at 95% confidence intervals (95% CI) were recorded for logistic regression analysis. All statistical analysis was performed on the Statistical Package for Social Sciences (SPSS), version 22. A two sided $p< 0.05$ was considered significant.

4.5 Results

The characteristics of the 505 participants comprising 264 controls and 241 cases are shown in **Table 4.1**. Over 44% of all T2DM patients had hypertension, with a male to female ratio of 98/142. Other demographic measures were overweight (33.19%), obese (18.26%), tertiary education (14.52%), moderate activity (67.21%), employed (55.17%), smoking history (14.10%) and alcohol history (42.32%).

Table 4.1 Characteristics of study participants with or without T2DM or hypertension (HPT)

Variable	Control	T2DM Only	T2DM+HPT	X^2	p-value
Age groups				27.75	**0.001**
31-40 years	14(5.3)	12(9.1)	2(1.9)		
41-50 years	31(11.7)	32(24.2)	17(15.7)		
51-60 years	74(28.0)	45(34.1)	36(33.3)		
61-70 years	87(33.0)	31(23.5)	37(34.3)		
71-80 years	58(22.0)	12(9.1)	16(14.8)		
Gender				0.985	0.611
Male	98(37.1)	52(39.4)	46(42.6)		
Female	166(62.9)	80(60.6)	62(57.4)		
BMI				15.39	**0.017**
Underweight	13(4.9)	8(6.1)	1(0.9)		
Normal weight	107(40.5)	67(51.1)	39(36.1)		
Overweight	87(33.0)	32(24.4)	48(44.4)		
Obese	57(21.6)	20(18.5)	24(18.3)		
Marital status				23.77	**0.003**
Married	174(65.9)	91(68.9)	72(66.7)		
Never married	29(11.0)	3(2.3)	1(0.9)		
Divorced	24(9.1)	12(9.1)	13(12.1)		
Widowed	37(14.0)	26(19.7)	22(20.4)		
Education				15.66	**0.048**
Tertiary	36(13.6)	17(12.9)	18(16.7)		
Senior High school	82(31.1)	38(28.8)	19(17.6)		
Junior high school	93(35.2)	38(28.8)	40(37.0)		
Lower primary	31(11.7)	26(12.1)	12(11.1)		
No formal education	22(8.3)	23(17.4)	19(17.6)		
Occupation				69.88	**0.0001**
Employed	107(40.5)	82(62.1)	51(47.2)		
Retired	23(8.7)	12(9.1)	22(20.4)		
Unemployed	32(12.2)	28(21.2)	23(21.3)		
Informal employment	102(38.6)	10(7.6)	12(11.1)		
Physical activity				11.07	0.086
Primarily sedentary	87(33.0)	35(26.5)	43(39.8)		
Moderate activity	177(67.5)	97(73.5)	65(60.1)		
Family history				54.59	**0.0001**
Yes	121(46.0)	97(73.5)	85(78.7)		
Smoking history				11.09	**0.026**
Yes	17(6.5)	17(12.9)	17(15.7)		
History of alcohol intake				9.57	**0.048**
Yes	83(31.7)	54(40.9)	48(44.4)		

Tests of significance were two tailed ($p<0.05$) and are bolded in the table.

Table 4.2 shows the clinical data of the participants. The mean age for T2DM only and T2DM with hypertension were 55.89 ± 11.27 and 60.07 ± 9.93, respectively whereas. BMI was not different between T2DM and hypertensive T2DM patients (p=0.158). In addition, WHR was higher among T2DM patients with hypertension (0.92 ± 0.55 vs 0.94 ± 0.061; p<0.0001). However, FPG, HbA1c, TC, TG, HDL-c, LDL-c, and CR were not different in T2DM and T2DM with hypertension (p>0.05). Further, T2DM patients were generally older than controls (p<0.0001), had a higher WHR (0.94 ± 0.061 vs 0.88 ± 0.08; p<0.0001), higher FPG (8.96 ± 4.18 vs 6.08 ± 1.79; p<0.0001) and higher HbA1c (8.23 ± 2.09 vs 5.45 ± 1.00; p<0.0001). However, compared with non-hypertensive T2DM patients, the controls had higher SBP (143.69 ± 25.82 vs 122.17 ± 11.86; p<0.0001), DBP (84.27 ± 15.37 vs 89.16 ± 12.62, P<0.0001) and CR (5.37 ± 1.49 vs 4.90 ± 1.52; p < 0.011). There were no differences in TC, TG, LDL-c, and VLDL-c between controls and non-hypertensive T2DM patients. Similarly, compared to controls, hypertensive T2DM patients were older (p<0.0001), had higher WHR's (0.94 ± 0.061 vs 0.88 ± 0.08 p<0.0001), higher SBP (160.48 ± 18.24 vs 84.27 ± 15.37; p<0.0001), and higher DBP (89.16 ± 12.62 vs 84.27 ± 15.37 p<0.0001).

Table 4.2 Clinical data of study participants with or without T2DM or hypertension

Variables	Controls	T2DM Only	T2DM+HPT	p-value
Age(years)	51.62 ± 11.92	55.89 ± 11.27†	60.07 ± 9.93*¥	**<0.0001**
BMI(kg/m^2)	25.86 ± 5.06	25.60 ± 5.38	26.80 ± 4.72	0.158
WHR	0.88 ± 0.08	0.92 ± 0.55†	0.94 ± 0.061*¥	**<0.0001**
SBP (mmHg)	143.69 ± 25.82	122.17 ± 11.86†	160.48 ± 18.24*¥	**<0.0001**
DBP (mmHg)	84.27 ± 15.37	75.45 ±11.29†	89.16 ± 12.62*¥	**<0.0001**
FPG (mmol/l)	6.08 ± 1.79	8.96 ± 4.18†	9.49 ± 4.68*	**<0.0001**
HbA1c (%)	5.45 ± 1.00	8.23 ± 2.09†	8.35 ± 2.09*	**<0.0001**
TC (mmol/l)	4.57 ± 1.25	4.71 ± 1.17	4.76 ± 1.39	0.342
TG (mmol/l)	1.32 ± 0.91	1.22 ± 0.57	1.33 ± 0.55	0.484
HDL-c (mmol/l)	1.23 ± 0.31	1.37 ± 0.35†	1.33 ± 0.29*	**<0.0001**
LDL-c (mmol/l)	2.77 ± 1.06	2.77 ± 1.11	2.81 ± 1.23	0.1
VLDL-c(mmol/l	0.59 ± 0.35	0.55 ± 0.26	0.60 ± 0.25	0.928
CR	5.37 ± 1.49	4.90 ± 1.52†	5.05 ± 1.53	**0.011**

Values are presented as mean ± SD. One-way ANOVA followed by Tukey Post hoc multiple comparison. BMI: p<0.05 was considered statistically significant. †p-value is significant (Comparison between control and DM only) *p-value is significant (Comparison between control and DM+HTN) ¥p-value is significant (Comparison between DM only and DM+HTN). Tests of statistical significance were two tailed (p<0.05) and are bolded in the table.

Table 4.3 outlines the gender stratification data of participants. The mean age of control participants was 51.67 ± 11.45 years with a male to female ratio of 98/166. A high proportion had at least a basic education (35.2%), were married (65.9%), and employed (40.5%). Women were generally obese compared to men when BMI (33.1% vs. 2.0%; p=0.001) and central adiposity (68.7% vs. 5.1%; p=0.001) were used respectively as an obesity index. A higher proportion of men than women were smokers (15.3% vs. 1.2%; p=0.001) and had a history of alcohol intake (41.8% vs. 25.6%; p=0.005). There was a significantly higher DBP (p=0.034), HbA1c (p=0.043), TC (p=0.001), HDL-c (p=0.011), non-HDL-c (p=0.004) and LDL-c (p=0.006) among women compared to men. Levels of SBP, FPG, TG, VLDL-c, CR and WHR among women were not significantly different from men (p>0.05).

Table 4.3 Characteristics of controls stratified by gender

Characteristics	Total	Men (n=98)	Women (n=166)	p-value
Age (years)	51.67 ± 11.45	51.09 ± 12.02	51.44 ± 11.89	0.761
Anthropometric data				
Waist to hip ratio	0.88 ± 0.07	0.89 ± 0.06	0.87 ± 0.08	0.148
Body mass index (kg/m^2)				<**0.0001**
Underweight	13(4.9)	8(8.2)	5(3.0)	
Normal weight	107(40.5)	60(61.2)	47(28.3)	
Overweight	87(33.0)	28(28.6)	59(35.5)	
Obese	57(21.6)	2(2.0)	55(33.1)	
Central obesity				<**0.0001**
Normal	145(54.9)	93(94.9)	52(31.3)	
Obese	119(45.1)	5(5.1)	114(68.7)	
Socio-economic data				
Education				<**0.0001**
Tertiary	36(13.6)	26(26.5)	10(6.0)	
Senior high school	82(31.1)	26(26.5)	56(33.7)	
Junior high school	93(35.2)	35(35.7)	58(34.9)	
Lower primary	31(11.7)	6(6.1)	25(15.1)	
No formal education	22(8.3)	5(5.1)	17(10.2)	
Marital status				**0.001**
Married	174(65.9)	75(76.5)	99(59.6)	
Never married	29(11.0)	14(14.3)	15(9.0)	
Divorced	24(9.1)	3(3.0)	21(12.6)	
Widowed	37(14.0)	6(6.1)	31(18.7)	
Occupation				<**0.001**
Employed	107(40.5)	52(53.1)	55(33.1)	
Retired	23(8.7)	13(13.3)	10(6.0)	
Unemployed	32(12.2)	1(1.0)	31(18.6)	
Informal employment	102(38.6)	32(32.7)	70(42.2)	
Biochemical data				
SBP (mmHg)	144.12 ± 26.61	145.82 ± 30.96	142.43 ± 22.25	0.305
DBP (mmHg)	83.74 ± 15.70	81.66 ± 18.02	85.81 ± 13.38	**0.034**
FPG (mmol/l)	6.08 ± 1.79	6.04 ± 1.78	6.11 ± 1.79	0.751
HbA1c (%)	5.41 ± 0.98	5.28 ± 0.91	5.54 ± 1.04	**0.043**
TC (mmol/l)	4.50 ± 1.17	4.24 ± 1.00	4.76 ± 1.33	**0.001**
TG (mmol/l)	1.29 ± 0.89	1.19 ± 0. 81	1.39 ± 0.96	0.105
HDL-c (mmol/l)	1.12 ± 0.30	1.16 ± 0.28	1.26 ± 0.32	**0.011**
NonHDL-c (mmol/l)	3.23 ± 1.09	3.07 ± 0.91	3.50 ± 1.26	**0.004**
VLDL-c (mmol/l)	0.58 ± 0.35	0.54 ± 0.36	0.61 ± 0.34	0.133
LDL-c (mmol/l)	2.73 ± 2.03	2.54 ± 0.91	2.91 ± 1.12	**0.006**
CR	5.33 ± 2.87	5.22 ± 1.28	5.45 ± 1.59	0.236
Family history and activity				
Diabetes family history (yes)	121 (46.0)	43(43.9%)	78(47.3%)	0.343
Smoking (yes)	17(6.5)	15(15.3)	2(1.2)	**<0.001**
Drinking (yes)	83(31.7)	41(41.8)	42(25.6)	**0.005**
Physical activity				**0.037**
Primarily sedentary	87(33.0)	29(29.6)	58(34.9)	
Moderate activity	135(51.1)	46(46.9)	89(53.6)	
Primarily physical	42(16)	23(23.4)	19(11.4)	

Data is expressed as mean ± standard deviation or (n %) and tests of significance were two tailed ($p<0.05$) and are bolded in the table.

Table 4.4 shows the distribution of clinical data of participants differing by SHS. With a median SHS score of 21, participants were grouped into high SHS (≥ 21) and low SHS (< 21). Gender (p=0.023), age (p=0.020), education (p=0.001), marital status (p=0.019), occupation (p<0.0001) and physical activity (p=0.006) were significantly associated with high SHS. Meanwhile, being a female [aOR=1.7(1.04-2.85); p=0.034)], elderly [aOR=10.8(1.69-68.97); p=0.018)], illiterate [aOR=5.34(1.61-17.77); p=0.007)], lower primary education [aOR=3.14(1.14-8.65); p=0.029)], widowed [aOR=2.75(1.28-5.91); p=0.011)], retired [aOR=7.0(2.40-20.40); p=0.0001)], unemployed [aOR=4.28(1.83-9.99); p=0.0009)], informal employment [aOR=2.68(1.52-4.68); p=0.0008)] and primarily sedentary [aOR=2.97(1.38-6.39); p=0.034)] were significant independent risk factors for high SHS after adjusting for age and gender. Participants with high SHS had a significantly higher mean SBP (p=0.004) and DBP (p=0.001) compared to those with low SHS. However, there were no significant differences between the mean lipid profile among participants with high SHS compared to low SHS (p>0.05).

Table 4.4 Distribution of factors with or without SHS

Variables	Total n (%)	SHS Score ≥21 n (%)	SHS score<21 n (%)	p-value	X^2	aOR (95%CI)	p
Gender				**0.023**	4.49		
Male	98(37.3)	42(31.1)	56(43.8)			1.0#	
Female	165(62.7)	93(68.9)	72(56.3)			1.7 (1.04-2.85)	**0.034**
Age (years)				**0.02**	13.34		
21-30	14(5.3)	5(3.7)	9(7.0)			1.0#	
31-40	30(11.4)	17(12.6)	13(10.2)			2.35(0.63-8.73)	0.332
41-50	74(28.1)	41(32.0)	33(24.4)			1.45(0.44-4.72)	0.574
51-60	87(33.1)	47(36.7)	40(29.6)			1.53(0.47-4.94)	0.569
61-70	44(16.7)	28(20.7)	16(12.5)			3.15(0.89-11.04)	0.119
71-80	14(5.3)	12(8.9)	2(1.6)			10.8(1.69-68.97)	**0.018**
Education				**0.001**	19.81		
Tertiary	36(13.7)	14(10.4)	22(17.2)			1.0#	
Senior high school	82(31.2)	30(22.2)	52(40.6)			0.91(0.40-2.03)	0.838
Junior high school	93(35.4)	54(40.0)	39(30.5)			2.17(0.99-4.78)	0.076
Lower primary	30(11.4)	20(14.8)	10(7.8)			3.14(1.14-8.65)	**0.029**
No education	22(8.4)	17(12.6)	5(3.9)			5.34(1.61-17.77)	**0.007**
Marital Status				**0.019**	11.76		
Married	173(68.5)	80(59.3)	93(72.7)			1.0#	
Never married	29(11.0)	13(9.6)	16(12.5)			0.94(0.42-2.08)	0.999
Divorced/separated	24(9.1)	16(11.8)	8(6.2)			2.32(0.94-5.72)	0.081
Widowed	37(14.1)	26(19.3)	11(8.6)			2.75(1.28-5.91)	**0.011**
Occupation				<**0.001**	27.09		
Employed	106(40.3)	36(26.7)	70(54.7)			1.0#	
Retired	23(8.7)	18(13.3)	5(3.9)			7.00(2.40-20.40)	**0.0001**
Unemployed	32(12.2)	22(16.3)	10(7.8)			4.28(1.83-9.99)	**0.0009**
Informal employment	102(38.8)	59(43.7)	43(33.6)			2.68(1.52-4.68)	**0.0008**
Physical activity				**0.006**	12.35		
Primarily sedentary	87(33.1)	57(42.2)	30(23.4)			2.97(1.38-6.39)	**0.007**
moderate activity	135(51.3)	62(45.9)	73(57)			1.32(0.65-2.71)	0.476
Primarily physical	41(15.6)	16(11.9)	25(19.6)			1.0#	
Biochemical data							
SBP (mmHg)	143.69 ± 25.82	148.33 ± 24.05	139.20 ± 26.58	**0.004**			
DBP (mmHg)	84.27 ± 15.37	87.33 ± 15.35	81.24 ± 14.7	**0.001**			
FBG (mmol/l)	6.08 ± 1.78	6.15 ± 1.75	6.01 ± 1.84	0.544			
HbA1c (%)	5.45 ± 0.99	5.44 ± 0.94	5.44 ± 1.06	0.997			
TC(mmol/l)	4.57 ± 1.25	4.66 ± 1.3	4.47 ± 1.18	0.217			
TG (mmol/l)	1.32 ± 0.91	1.41 ± 1.01	1.22 ± 0.79	0.099			
HDL-C (mmol/l)	1.23 ± 0.31	1.25 ± 0.32	1.20 ± 0.3	0.177			
VLDL-C (mmol/l)	0.59 ± 0.35	0.62 ± 0.35	0.56 ± 0.36	0.155			
LDL-C (mmol/l)	2.77 ± 1.06	2.81 ± 1.09	2.73 ± 1.02	0.554			
CR	5.37 ± 1.49	5.40 ± 1.5	5.35 ± 1.47	0.805			

aOR: adjusted odds ratio, CI confidence interval. Multivariate regression model was adjusted for age and gender; #: reference, $p < 0.05$. Tests of significance were two tailed ($p<0.05$).

Table 4.5 shows that after adjusting for age and gender, central adiposity [aOR=1.74(1.06-2.83); p=0.027)], underweight [aOR=5.82(1.23-27.52); p=0.018)], high SBP [aOR=1.86(1.14-3.05); p=0.012)], high DBP [aOR=2.39(1.40-4.07); p=0.001)] and high TG [aOR=2.17(1.09-4.33); p=0.029)] were found to be significant independent risk factors associated with high SHS.

Table 4.5 Association between SHS and metabolic risk factors

Variables	Total (n %)	SHS Score ≥21 (n %)	SHS score<21 (n %)	x^2	p	OR (95%CI)	P
Central Obesity					**0.018**		
Normal	144(54.8)	65(48.1)	79(61.7)			1.0#	
Obese	119(45.2)	70(51.9)	49(38.3)			1.74 (1.06-2.83)	**0.027**
BMI				6.75	0.08		
Underweight	13(4.9)	11(8.1)	2(1.6)			5.82(1.23-27.52)	**0.018**
Normal weight	107(40.7)	52(38.5)	55(43.0)			1.0#	
Overweight	86(32.7)	41(30.4)	45 (35.2)			0.96(0.54-1.70)	0.987
Obese	57(21.7)	31(11.8)	26(20.3)			1.26(0.66-2.40)	0.514
Blood Pressure							
SBP							
Normal SBP	121(46.0)	52(38.5)	69(53.9)	6.26	**0.009**	1.0#	
High SBP	142(54.0)	83(61.5)	59(46.1)			1.86(1.14-3.05)	**0.012**
DBP							
Normal SBP	176(66.9)	78(57.8)	98(76.6)	10.47	**0.001**	1.0#	
High SBP	87(33.1)	57(42.2)	30(23.4)			2.39(1.40-4.07)	**0.001**
FPG				2.87	0.090		
Normal	113(43.1)	51(38.1)	62(48.4)			1.0#	
High	149(56.9)	83(61.9)	66(51.6)			0.65(0.40-1.07)	0.105
HbA1c				1.93	0.164		
Normal	195(75.6)	98(73.7)	97(77.6)			1.0#	
High	63 (24.4)	38(29.2)	25(19.5)			1.50(0.84-2.86)	0.192
TC				0.03	0.489		
Normal	186(72.1)	96(71.6)	90(72.6)			1.0#	
High	72(27.9)	38(28.4)	34(27.4)			1.05(0.61-1.81)	0.867
TG				4.97	**0.03**		
Normal	215(83.3)	105(78.4)	110(88.7)			1.0#	
High	43(16.7)	29(21.6)	14(11.3)			2.17(1.09-4.33)	**0.029**
HDL-c							
Normal	151(58.5)	78(58.2)	73(58.9)	0.12	0.508	1.0#	
Low	107(41.5)	56(41.8)	51(41.1)			1.03(0.63-1.69)	0.999
Non-HDL-c				1.66	0.123		
Normal	137(53.1)	66(49.3)	71(57.3)			1.0#	
High	121(46.9)	53(42.7)	68(50.7)			1.38(0.85-2.25)	0.532
LDL-c							
Normal	126(48.8)	65(48.5)	61(49.2)	0.12	0.506	1.0#	
High	132(51.2)	69(51.5)	63(50.8)			1.03(0.63-1.68)	0.999

aOR: adjusted odds ratio, CI confidence interval. #: reference, $p < 0.05$. Tests of significance were two tailed ($p<0.05$) and are bolded.

Table 4.6 demonstrates that after controlling for age and gender, significant positive linear relationships were observed between SHS score and SBP, DBP and CR irrespective of gender ($p < 0.05$). There were inconsistent correlations between other risk factors and high SHS. SHS scores were significantly associated with LDL-c in men while FPG, TC and non-HDL-c were significantly associated with SHS in women ($p<0.05$). There was no significant linear relationship between BMI, HbA1c, TG, HDL-c, VLDL-c and high SHS in either men or women ($p>0.05$).

Table 4.6 Multivariate linear regression model for SHS score in relation to metabolic risk factors stratified by sex

	MALE			FEMALE		
	β	SE	p-value	β	SE	p
BMI	0.52	0.44	0.237	0.16	0.22	0.454
SBP	2.09	0.51	**0.035**	1.52	0.05	**0.046**
DBP	2.16	0.84	**0.012**	2.11	0.07	**0.005**
FPG	0.67	0.86	0.442	2.09	0.62	**0.0009**
HbA1c	0.13	1.68	0.936	1.17	1.1	0.118
TC	1.33	1.52	0.387	1.75	0.85	**0.043**
TG	2.07	1.89	0.276	1.18	1.2	0.329
HDL-C	-9.27	5.32	0.085	-1.42	3.64	0.696
Non-HDL	2.56	1.68	0.131	2.08	0.91	**0.024**
VLDL	4.60	4.16	0.272	4.19	3.39	0.219
LDL-C	3.31	1.66	**0.049**	2.02	1.02	0.051
CR	2.79	1.16	**0.019**	1.45	0.72	**0.049**

β: regression coefficient; SE: Standard error. Tests of significance were two tailed ($p<0.05$) and are bold.

4.6 Discussion

T2DM is largely a consequence of accumulating metabolic damage due to increasing urbanisation, physical inactivity, unhealthy eating and sedentary lifestyle (Bi et al., 2012; Chen et al., 2012; Rich et al., 2013; Stumvoll, Goldstein, & van Haeften, 2005). Earlier diagnosis remains the blueprint for preventing T2DM and promoting better health outcomes (Deepa, Anjana, & Mohan, 2017; DeFronzo & Abdul-Ghani, 2011; Hulsegge et al., 2017; Zou et al., 2017). This study is premised on the hypothesis that cardiometabolic risk factors are prevalent in Kumasi, an urban city in Ghana (Roberts, et al., 2017). As such, we have explored modifiable risk factors in both T2DM sufferers and healthy controls **(Tables 4.1, 4.2 & 4.3)**.

Among the controls, we used a simple and inexpensive tool (SHSQ25) to determine highly at-risk individuals. Participants were classified into two groups based on how they rated on the SHSQ25. Here, a median score <21 represents low SHS (good health)

whereas a median score >21 represents high SHS (poor health). Ideally, filling in this short questionnaire alone should encourage individuals who obtain a high SHS score to have their clinical/biochemical indicators measured. Health providers on dietary/lifestyle modifications that will enable them to live healthier and delay the onset of T2DM could advise such persons. Alternatively, a person with a high SHS may have undiagnosed, asymptomatic T2DM, or its related co-morbidities and may need immediate intervention or therapy.

In this study, undiagnosed hypertension is prevalent, and similar to previous findings, high SHS is significantly associated with both DBP and SBP **(Tables 4.4 & 4.6)**. This also confirms the findings of another community-based study in the subregion, which showed that a high proportion of adults in Sub-Saharan Africa (SSA) (44-93%) who have high blood pressure are unaware of it (Cappuccio & Miller, 2016; Echouffo-Tcheugui, Kengne, Erqou, & Cooper, 2015). Yet another study in a peri-urban community in Ghana showed the prevalence of undiagnosed hypertension at 28.7% (Cappuccio & Miller, 2016). This is in fact disturbing because high BP is by far the main risk factor for T2DM and cardiovascular disease (CVD) (Cappuccio & Miller, 2016; Mendis, Puska, & Norrving, 2011; Ofori-Asenso & Garcia, 2016). High BP, for example, causes 42% of all ischaemic heart diseases (Mensah, 2008) and one-third of all heart failures (Khatibzadeh et al., 2013). As such, there is an overarching need to identify these individuals and begin treatment to avoid complicated health outcomes.

Similar to previous findings, age was associated with high SHS **(Table 4.4)**. This is not surprising since ageing is associated with less physical activity and sedentary lifestyle, making it a high order independent risk factor for T2DM (Yu et al., 2016). From the perspective of metabolism, this age range is accompanied by an imbalance in the production of reactive oxygen species (ROS) and inflammation that together lead to metabolic dysregulation. Metabolic dysregulation will lead to insulin resistance and consequently T2DM (Franco et al., 2009).

In addition, gender, education, marital status, occupation and physical activity were associated with high SHS (**Table 4.4**). However, we could not validate the association between high SHS and higher FPG, HbA1c, TC, LDL-c and Low HDL-c **(Table 4.5)**. In part, this observation could be attributed to the small sample size used for this investigation. All previous investigations involved large cohorts in China i.e. 2,799 participants in 2009 (Yan et al., 2009), 3,019 in 2012 (Yan et al., 2012), 3,405 in 2012 (Wang & Yan, 2012) and 4,313 in 2016 (Wang et al., 2016). Cohorts from geographically

distinct populations are exposed to different stressors (e.g. variation in job types, lifestyles, and socioeconomic, environmental and cultural factors). For example, while the majority of the Ghanaian participants are primarily sedentary and engage in less energy demanding jobs, the Chinese cohorts are mainly industry workers who spend long hours at work, and are therefore more likely be stressed. Consequently, these disparities in stressful conditions, especially in the hours preceding testing, may affect biochemical assessments. Further, it is possible that the biochemical assessments of this present study are somewhat influenced by laboratory conditions (Bonora & Tuomilehto, 2011). Therefore, other highly sensitive and state-of-the art health facilities should be available to enable validation.

Among T2DM sufferers, hypertension was high and this agrees with a previous study reported from the Kumasi region (**Table 4.2**) (Danquah et al., 2012). Further, the results of the present study show that the majority of T2DM patients had FPG and HbA1c levels higher than the recommended targets (i.e. > 7 and > 7.2 respectively), many of whom are on the path to developing complications and co-morbidities. Surprisingly, all these individuals have been provided with both blood pressure lowering and lipid lowering medications. This could be attributed to delayed intervention, ineffective treatments, untargeted medications, and drug response and drug resistance. Alternatively, the suboptimal management could be due to other factors including: 1) Institutional (e.g., health care policies, facilities and resources), 2) Environmental, dietary and lifestyles 3) Genetic and epigenetics and 4) Individual factors (physical, mental, social and spiritual wellbeing). In order to address such a complex situation, there must be a transition from the current medical practise to PPPM. PPPM holds the key to revolutionising T2DM care by promoting adequate patient stratification, disease modelling, surveillance, optimal diagnosis and prediction of adverse drug-drug interactions (Golubnitschaja, 2010; Golubnitschaja et al., 2016; Golubnitschaja et al., 2014; Lemke & Golubnitschaja, 2014). Taken together, this will lead to better health outcomes, delay the onset of complications, improve quality of life and promote longevity.

Overall, it is clear that modifiable risk factors are prevalent among T2DM sufferers but importantly, it is demonstrated that SHSQ-25 could be a risk stratification tool for T2DM. Compared to many survey instruments and risk prediction models (Liu et al., 2011; Mehrabi et al., 2010; Rathmann et al., 2010), the SHSQ-25 is simple, inexpensive and can be self-completed prior to, or administered during, a consultation. The scoring system is easy and data interpretation/analysis does not require special expertise to

perform. Whilst recognising this, this tool is a subjective health measure and it should be supported with advanced objective biomarkers. Currently, highly sophisticated and powerful analytical tools are available for measuring, detecting and characterising important biomarkers (Adua et al., 2017; Wang, 2016; Yu et al., 2016). This will assist in the understanding of the molecular intricacies that underpin the disease' pathogenesis. For example, it is possible to determine transcriptional regulation, post-translational modifications, protein expression and interaction and altered enzyme activity (Adua, Russell, et al., 2017; Wang, 2016). Research has examined N-glycosylation profiles in metabolic syndrome (MetS) (Lu et al., 2011). It was shown that nine N-glycan traits were associated with DBP, SBP, FPG and BMI and these could be potential biomarkers for MetS (Lu et al., 2011).

Before concluding, some limitations need to be mentioned. The major one is related to the cross-sectional design. There was an inability to determine the proportion of participants in the high SHS group who will develop T2DM over time. The study tried to perform age-gender matching but the recruited controls were still generally younger than cases. However, this does not invalidate the significance of the findings of this study since potential confounding was somewhat addressed by logistic regression and multivariate analyses. The sample size of the study does not allow a generalisation to be made. Moreover, metabolic risk factors such as blood pressure, blood glucose and lipid profiles, particularly among the controls were limited to only one time measurement and therefore the prevalence of risk factors may be under or overestimated.

4.7 Conclusion

There is poor management of risk factors among T2DM patients in this region of Ghana. More disturbing is the fact that the majority of people who are at risk, particularly of hypertension, are undiagnosed. This underscores the need for novel screening tools that can reveal such individuals. The SHSQ-25 represents an instrument of choice and in turn sets the platform for prediction, prevention and treatment of T2DM, which is vital, particularly for a region where laboratory-based measures are not routinely available.

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Chapter 5 is not available in this version of the thesis

CHAPTER SIX

Prelude

Study II (Chapter Five) showed that MetS was associated with an increase in plasma concentration of glycoproteins predominately carrying N-glycans. In this study, MetS was established based on the recognition of three risk factors: high blood pressure, high TG, LDL-c, FPG levels and central obesity. However, it was necessary to extend this research by profiling N-glycan structures in clinically diagnosed T2DM patients as per the ICD criteria. Further, it was crucial to examine how the structures in T2DM differ from aged-gender matched controls **(Study III)**. This manuscript has been published in the *Journal of Archives of Biochemistry and Biophysics* and it can be cited as:

Adua E, Memarian E, Russell A, Trbojević-Akmačić I, Gudelj I, Jurić J, Roberts P, Lauc G, Wang W (2018). High throughput profiling of whole plasma N-glycans in type II diabetes mellitus patients and healthy individuals: A perspective from a Ghanaian population. *Archives of biochemistry and biophysics Arch Biochem Biophys.* 661:10-21.

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High-throughput profiling of whole plasma N-glycans in Type II diabetes mellitus patients and healthy individuals: A perspective from a Ghanaian population

6.1 Abstract

Aberrant protein glycosylation may reflect changes in cell metabolism of type II diabetes mellitus (T2DM) and offers fresh vistas for discovering potential biomarkers. However, the functional significance of T2DM N-glycan alterations is underexplored, since to date N-glycan profiling studies have been mainly performed in selected populations. Geographically and genetically isolated populations are needed for validation of specific biomarkers. From January to June 2016, an age-gender matched cross-sectional study comprising 232 T2DM patients and 219 controls was conducted in Ghana, Western Africa. Blood plasma samples were collected for clinical assessment after which plasma N-glycans were freed, fluorescently labelled and analysed by ultra-performance liquid chromatography (UPLC). Statistical analyses were performed and false discovery rate (FDR) controlled by the Benjamini- Hochberg method. High branching (HB) [W= 46328; q=0.00072], tri-galactosylated (G3) [W= 44076; q=0.00096], antennary fucosylated (FUC_A) [W=43055; q= 0.0000763], and triantennary (TRIA) [W= 44624; q=0.0025], N-glycan structures were increased in T2DM whereas low branching (LB) [W= 46328; q=0.00072], non-sialylated (S0) [W= 46929; q=0.00292], monogalactosylation (G1) [W= 44091; q=0.0000763], core fucosylation (FUC_C), [W= 46497; q=0.00096], biantennary galactosylation (A2G) [W= 45663; q=0.000763], and biantennary (BA) [W= 46376; q=0.00072], structures were decreased compared to controls. Nine N-glycan peaks (GPs (GP1, GP4, GP7, GP11, GP17, GP19, GP22, GP26, GP29)) were found to predict case status based on Akaike's information criterion (AIC) and Bayesian information criterion (BIC) model selection. Adjusting for age, sex and other co-variates in this model yielded an area under the curve (AUC) of 80.5% with sensitivity of 79% and specificity of 73%, indicating the predicting power of N-glycans as robust biomarkers. Our results show that hyperglycemia influences N-glycan complexities among Ghanaians. N-glycan profiling in distinct populations has affirmed the potentiality of N-glycan profiles as generic biomarkers, which may facilitate better prognosis for T2DM.

Key words: biomarker, N-glycan, type II diabetes mellitus

6.2 Introduction

Type II diabetes mellitus (T2DM) is a major health challenge worldwide, responsible for much mortality and morbidity (International Diabetes Federation, 2015; The World Health Organisation, 2014; Zilliox, Chadrasekaran, Kwan, & Russell, 2016). In fact, over one million deaths in 2015 were attributed to T2DM while the disease prevalence is still rising (International Diabetes Federation, 2015; WHO, 2015b). Additionally, those who survive it experience the debilitating effects of co-morbidities that can lead to a decreased quality of life and productivity (Zhong et al., 2015), as well as huge financial burden because of frequent hospitalisation and significant medication costs (Lim et al., 2013; WHO, 2015a). To date, T2DM has no cure but is only managed with disease-modifying drugs (Adua et al., 2017; Bolen et al., 2007; Chaudhury et al., 2017; Reusch & Manson, 2017). This is primarily because the aetiology or the pathophysiology of T2DM is still obscure and complex, with many researchers attributing its complexity to the combined effects of genetic epigenetic and environmental factors (Grarup, Sandholt, Hansen, & Pedersen, 2014; Morris et al., 2012). However, complex oligosaccharides (glycans) represent the intermediary between our genetic make-up and the cellular environment (Knežević et al., 2010; Lauc, 2016; National Research Council (US) Committee on Assessing the Importance and Impact of Glycomics and Glycosciences, 2012; Raman et al., 2005; Russell et al., 2018).

Glycans bind to protein backbones in a process termed glycosylation and thus far, it is regarded as the most complex and abundant co- and post-translational process in the cell (Varki, 2009). Protein glycosylation is vital for many biological functions including cell recognition and signalling, localisation, immune response and cellular regulation (Varki, 2009; Cummings & Pierce, 2014; Wang, 2016). Protein glycosylation can be O-linked, glycosaminoglycan (GAG), C-linked and N-linked glycans, the latter being the most studied and well understood (Varki, 2009; Cummings & Pierce, 2009). N-glycans are a subclass of glycan types that bind to asparagine side chains of proteins in the consensus sequence Asn-X-Thr/Ser (where X is any amino acid except proline) (Varki et al., 2009). Although these structures are fairly stable within an individual they change under the influence of an external perturbation, with different physiological parameters such as age (Gornik et al., 2009; Knezevic et al., 2008), sex (Baković et al., 2013) and pathophysiological conditions such as rheumatoid arthritis (Bondt et al., 2018; Gudelj et al., 2018), cancers (Lauc et al., 2013; Theodoratou et al., 2016; Vuckovic et al., 2015),

Parkinson's disease (Russell et al., 2017), Alzheimer's disease (Frenkel-Pinter et al., 2017), metabolic syndrome (Lu et al., 2011) and T2DM (Lauc, 2016; Campbell, 2016).

However, aberrant glycosylation in T2DM has only been reported in a few studies (Itoh et al., 2007; Keser et al., 2017; Lemmers et al., 2017) but these have indicated the functional significance of certain N-glycan structures in T2DM. For example, it was hypothesised in the early 1970's that increased serum fucose was associated with diabetes (McMillan, 1972). This was confirmed by Itoh et al., (2007) who suggested a link between increased α-1,6-linked fucose with T2DM in the sera of db/db mice and humans (Itoh et al., 2007). Both of these studies were largely limited by sample size, suboptimal research design and lack of powerful analytical tools. After nearly four decades, Testa et al., (2015) performed a large-scale study on Caucasians and showed that core-fucosylated diantennary and α-1, 6-linked arm monogalactosylated N-glycans were reduced in T2DM compared to controls. However, since DNA sequencer-aided flourophore-assisted carbohydrate electrophophoresis (DSA-FACE) was the tool employed, they could not measure sialylation and the inadequate resolution resulted in quantification of only the ten most abundant N-glycan peaks (Testa et al., 2015).

Advances in technology have allowed an effective and better large-scale N-glycan characterisation and analysis in a high throughput manner. Among the recently developed analytical tools including nuclear magnetic resonance (NMR) imaging, capillary electrophoresis (Schwedler, 2014), liquid chromatography (Royle et al., 2008), matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS)(Wuhrer, 2013) and ultra-performance liquid chromatography (UPLC), the latter has emerged as a powerful and widely accepted tool for N-glycosylation analyses because it can efficiently separate N-glycan isomers, it is cost effective, reliable and has robust quantification (Bones et al., 2010; Trbojevic-Akmacic, Vilaj, & Lauc, 2016). Utilising this technique, Lemmers et al., (2017) investigated the immunoglobulin G (IgG) glycosylation patterns among independent European populations of T2DM individuals and healthy controls (Lemmers et al., 2017). Although this study provided insights into molecular mechanisms in the disease's pathophysiology, it was restricted to specific immunoglobulin glycans. As such, their attempt to delineate the molecular basis of T2DM was limited. The need for a total human plasma N-glycome analysis is thus warranted since such efforts will not only represent N-glycosylation alteration in one protein but all proteins in circulation.

After reviewing the literature, this is the first attempt to apply hydrophilic interaction liquid chromatography (HILIC) UPLC technology for a comprehensive N-

glycan profiling of T2DM patients in a geographically and genetically isolated population: sub-Saharan Africa (SSA), Ghana. The outcome of this study will elucidate the molecular underpinnings of plasma N-glycosylation in T2DM, which in turn, will provide clues for early diagnosis, prognosis and amplify therapeutic opportunities.

6.3 Methods and Study design

In this cross-sectional study, we recruited 232 T2DM and 219 age-gender matched healthy controls from January to July 2016. T2DM individuals were purposively sampled from the Diabetic Unit of the Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana whereas the controls were recruited by convenient sampling from three suburbs within the Kumasi metropolis (Ash-town, Pankrono and Abrepo). The study was reviewed and approved as described in **Chapter Four** of this thesis.

Inclusion Criteria

T2DM was established based on the international classification of disease 10 (ICD-10) criteria and known history of anti-diabetes medication use. The controls however, were individuals who were not suffering from T2DM and/or hypertension and had no history of anti-diabetes or antihypertensive medication use. In both groups, we excluded participants who were suffering from other chronic diseases related to the genitourinary, digestive, respiratory and haematological systems. The age range for all participants was 30-80 years.

Anthropometric Examination

Anthropometric measurements including weight, height, BMI, WHR, WHtR, SBP and DBP were measured by standard methods (**See Chapter Four** for detailed description).

Clinical Data

Details of this section are provided in **Chapter Four** of this thesis. Briefly, venous fasting blood samples were collected from each participant into tubes containing EDTA (ethylene diamine tetraacetic acid), fluoride oxalate and gel separator. Different clinical tests including FPG, HbA1c, TC, HDL-c, LDL-c, TG and VLDL-c were measured on the automated chemistry analyser (Roche Diagnostics, COBAS INTEGRA 400 Plus, USA). WHtR was then calculated. Aliquots of processed plasma samples were stored at -80^{o}C until N-glycan analysis.

N-glycan Analysis

Whole plasma N-glycoprofiling was possible with HILIC-UPLC technique and details of this experiment are described in **Chapter Five**. Briefly, N-glycans in 5µl plasma were released by digestion with peptide *N*-glycosidase F (ProZyme, USA), and labelled with 2-aminobenzamide (2AB) (Ludger Tag 2-AB labelling kit, UK). After incubation and washing steps, labelled N-glycans were separated by HILIC-UPLC instrument into 39 N-glycan peaks (GPs) **(Figure 6.1)**. Eleven derived traits were then calculated from the 39 peaks. Data processing was performed using an automated system and a dedicated software.

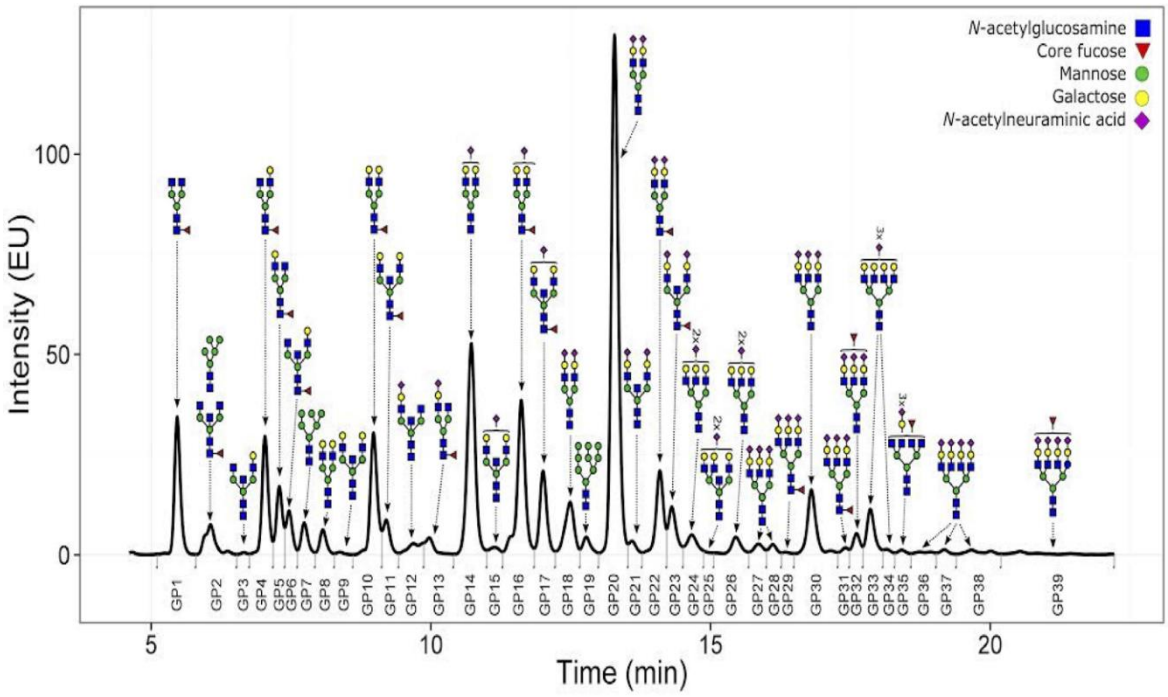

Figure 6.1. A chromatogram of 39 fluorescently labelled glycan peaks from the HILIC-UPLC. Reproduced with permission from Genos Glycoscience Research Laboratory, Zagreb, Croatia.

6.4 Statistical Analysis

Prior to statistical analysis, normalisation and batch correction on the UPLC data was performed in order to control for non-biological variability. Normality distribution of data was checked by the Kolmogov Smirnoff test as well as visualisation of QQ plots. All continuous data was recorded as mean ± standard deviation (Mean ± SD) while categorical variables were expressed as frequencies (percentages). However, because of the skewed nature of N-glycan data, interquartile ranges (IQR) were used to describe the data, hence they were presented as a median depending on the normality distribution,

between groups, comparisons for continuous variables were performed using Mann-Whitney U-tests or Student-t tests and intergroup comparisons of categorical variables were performed using Chi-square tests. The Spearman correlation method was used to calculate the correlation coefficients (rho) between biochemical parameters and N-glycans. The association between N-glycans and age in both males and females for cases and controls were determined by linear regression. To adjust for multiple testing, the Benjamini-Hochberg (BH) method was used to control the false discovery rate (FDR) (q). Prior to logistic regression, the data was rank transformed. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 23 and the R statistical package software version 3.4.3 (R Core team, 2017). Here, q-value is used to represent the p-value after correction for multiple testing and hence a q-value <0.05 was considered significant.

6.5 Results

Demographic and Biochemical Characteristics

The mean age for the participants was 56.54 ± 9.89 and 55.10 ± 9.27 years for cases and controls respectively, with their ages ranging from 30 to 80 years. The number of females was generally higher than males in both groups (i.e. 61.4% females in controls and 57.3% females in cases) but it was not statistically significant. A large proportion of participants in both groups were employed (χ^2=26.74, q=0.0003) and educated (χ^2=9.83, q=0.0812). Compared to controls, T2DM patients were primarily sedentary (χ^2=9.77, q=0.0446) whereas there was no statistical difference in BMI in both groups. In the clinical assessment, T2DM patients had higher FPG (U=9871.5; q=0.0001) and HDL-c (U=17868, q=0.0010) than controls. However, SBP (U=20863.5, q=0.0084) was higher in the controls compared to those with T2DM. WHtR (U=24057, q=0.5999), TC (U=21918; q=0.9604), TG (U=22012, q=0.9050) and LDL-c (U=20545, q=0.3322) were not statistically different in the groups **(Table 6.1)**.

Table 6.1 Characteristics of participants with and without T2DM.

Variable		Control	Case	Statistic	p	q
Age (mean ± SD)		55.10 ± 9.27	56.54 ± 9.89	-1.466[t]	0.0648	0.1102
Age (years)						
	31-40 years	8 (3.7)	14(6.0)	8.57^	0.073	0.1128
	41-50 years	70(32.0)	50(21.6			
	51-60 years	83(37.9)	87(37.5)			
	61-70 years	44(20.1)	63(27.2)			
	71-80 years	14(6.4)	18(7.8)			
Gender						
	Female	135 (61.4)	133 (57.30)			
BMI (Kg/m2)				1.302^	0.729	0.8262
	Underweight	11(5.0)	7(3)			
	Normal	91(41.6)	102(44.2)			
	Overweight	74(33.8)	77(33.0)			
	Obese	43(19.6)	45(19.5)			
Education				9.838^	0.043	0.0812
	Tertiary	29(13.3)	40(17.2)			
	Senior high	72(33.0)	53(22.8)			
	Junior high	71(32.6)	76(32.8)			
	Lower primary	28(12.8)	28(12.1)			
	No formal education	18(8.3)	35(15.1)			
Occupation				26.743^	0.0001*	**0.0003****
	Employed	147(67.4)	152(65.8)			
	Retired	21(9.6)	27(11.7)			
	Keeping house	14(6.4)	23(10.0)			
	Unemployed	26(16.6)	29(12.5)			
Physical activity						
	Sedentary	30(13.8)	53(22.9)	9.772^	0.021	**0.0446****
	Moderate activity	114(52.3)	94(40.7)			
	Active	74(34.0)	84(36.3)			
Clinical/biochemical data						
WHtR		0.56 ± 0.08	0.56 ± 0.08	24057[u]	0.4933	0.599
SBP (mmHg)		145.96 ± 24.3	139.78 ± 24.91	20863.5[u]	0.0035*	**0.0084****
DBP (mmHg)		84.70 ± 14.42	82.52 ± 13.10	22652[u]	0.0925	0.131
FPG (mmol/l)		5.86 ± 0.95	9.24 ± 4.26	9871.5[u]	0.0000*	**0.0001****
TC(mmol/l)		4.69 ± 1.26	4.66 ± 1.26	21918.5[u]	0.9604	0.9604
TG(mmol/l)		1.35 ± 0.97	1.24 ± 0.54	22012.5[u]	0.8518	0.905
HDL-c(mmol/l)		1.24 ± 0.33	1.35 ± 0.33	17868[u]	0.0003*	**0.0010****
LDL-c(mmol/l)		2.88 ± 1.05	2.74 ± 1.16	20545.5[u]	0.254	0.3322
CR		5.47 ± 1.50	4.90 ± 1.52	17132[u]	0.0001*	**0.0003****

Data presented as Mean ± SD and n (%). ^χ^2 test of independence, [t] Student's t-test, [u] Mann Whitney U tests. Tests of significance were two tailed (*p <0.05); **q<0.05 significant after correction for FDR and are bold.

Differential plasma N-glycan patterns in T2DM and healthy controls

The median interquartile ranges of all measured N-glycans are shown in **Table 6.2a** and obviously, there were distinct levels of N-glycans between cases and controls. Generally, GP4 (FA2[6]G1), GP5(FA2[3]G1), GP6(FA2[6]BG1), GP10(FA2G2), GP11(FA2BG2), GP13(FA2[3]G1S[3]1), GP16(FA2G2S[6]1), GP17(FA2BG2S[3]1), GP18(A2G2S[3,6]2) and GP29(FA3G3S[3,3,3]3 were higher among the controls compared to T2DM patients. In contrast, GP14(A2G2S[6]1, GP24(A3G3S[3,6]2), GP26(A3G3S[3,3]2), GP30(A3G3S[3,3,6]3) GP31(FA3G3S[3,3,6]3),

GP32(A3F1G3S[3,3,3]3), GP34(A4G4S[3,3,6]3) and GP36 (A4G4S[3,3,3,3]4) were higher in T2DM compared to controls (q<0.05).

Table 6.2a. Distribution of plasma N-glycan peak levels in cases and controls

	Control		Case				
	Median (IQR)	Range	Median (IQR)	range	W	P	q
GP1	6.34(2.61)	5.04 - 7.66	6.44(2.80)	5.07 - 7.88	48518	0.6364	0.68106
GP2	2.36(0.73)	2.05 - 2.78	2.41(0.82)	2.04 - 2.87	48568	0.6627	0.69698
GP3	0.09(0.048)	0.07 - 0.12	0.08(0.04)	0.07 - 0.11	48807	0.0574	0.09726
GP4	5.21(1.67)	4.34 - 6.01	4.44(1.40)	3.85 - 5.26	43677	1.00E-05	**7.63E-05****
GP5	2.16(0.71)	1.76 - 2.47	1.84(0.65)	1.54 - 2.19	44732	1.00E-05	**7.63E-05****
GP6	1.28(0.42)	1.09 - 1.51	1.20(0.39)	1.02 - 1.42	47514	0.0045	**0.01056****
GP7	0.97(0.19)	0.89 - 1.09	0.99(0.18)	0.90 - 1.08	48644	0.7034	0.72671
GP8	1.13(0.29)	1.01 - 1.31	1.12(0.32)	0.99 - 1.31	50910	0.7148	0.72671
GP9	0.10(0.03)	0.09 - 0.12	0.09(0.03)	0.09 - 0.12	49516	0.1667	0.25422
GP10	4.39(1.54)	3.47 - 5.02	3.69(1.45)	3.07 - 4.52	44785	1.00E-05	7.63E-05
GP11	0.79(0.22)	0.68 - 0.91	0.73(0.20)	0.62 - 0.83	46026	1.00E-04	**0.00051****
GP12	0.98(0.16)	0.90 - 1.06	0.99(0.18)	0.91 - 1.09	47517	0.2288	0.3323
GP13	0.85(0.28)	0.72 – 1.00	0.81(0.21)	0.71 - 0.92	48047	0.0141	**0.02775****
GP14	10.46(1.53)	9.69 - 11.23	10.87(1.54)	10.05 - 11.59	44881	0.0018	**0.00458****
GP15	0.37(0.08)	0.34 - 0.43	0.37(0.10)	0.34 - 0.44	48788	0.7829	0.7829
GP16	5.96(1.50)	5.32 - 6.82	5.66(1.39)	5.03 - 6.42	46690	6.00E-04	**0.00174****
GP17	1.74(0.55)	1.46 - 2.01	1.55(0.55)	1.32 - 1.88	46047	1.00E-04	**0.00051****
GP18	3.52(0.71)	3.18 - 3.90	3.40(0.69)	3.05 - 3.75	47842	0.0092	**0.02004****
GP19	1.11(0.21)	1.01 - 1.22	1.11(0.21)	1.00 - 1.21	50554	0.5318	0.6161
GP20	25.01(3.49)	23.26 - 26.76	25.66(3.90)	23.65 - 27.55	46313	0.0373	0.06895
GP21	0.51(0.13)	0.46 - 0.59	0.51(0.17)	0.44 - 0.61	49992	0.3004	0.38988
GP22	4.17(0.97)	3.79 - 4.77	4.31(1.05)	3.89 - 4.95	46308	0.037	0.06895
GP23	1.99(0.64)	1.71 - 2.35	1.90(0.66)	1.61 - 2.28	49112	0.0934	0.15031
GP24	1.63(0.55)	1.35 - 1.90	1.74(0.71)	1.38 - 2.10	45553	0.0084	**0.01898****
GP25	0.14(0.05)	0.12 - 0.17	0.15(0.06)	0.13 - 0.19	46439	0.0466	0.08361
GP26	1.47(0.41)	1.27 - 1.69	1.64(0.53)	1.37 - 1.91	42630	1.00E-05	**7.63E-05****
GP27	0.45(0.32)	0.31 - 0.63	0.47(0.38)	0.30 - 0.68	48488	0.6209	0.67634
GP28	0.77(0.27)	0.64 - 0.92	0.80(0.32)	0.63 - 0.96	47671	0.2753	0.36507
GP29	0.20(0.06)	0.18 - 0.24	0.19(0.05)	0.16 - 0.22	46155	1.00E-04	**0.00051****
GP30	5.60(1.84)	4.50 - 6.34	5.89(2.20)	4.70 - 6.91	45661	0.0105	**0.02209****
GP31	0.47(0.20)	0.37 - 0.57	0.50(0.27)	0.41 - 0.69	44950	0.0021	**0.00512****
GP32	1.31(0.48)	1.09 - 1.57	1.46(0.57)	1.24 - 1.82	42435	1.00E-05	**7.63E-05****
GP33	1.79(1.28)	1.23 - 2.52	1.88(1.57)	1.15 - 2.73	48349	0.5511	0.6161
GP34	0.36(0.09)	0.32 - 0.41	0.39(0.13)	0.33 - 0.47	43628	1.00E-04	**0.00051****
GP35	0.24(0.13)	0.18 - 0.32	0.26(0.16)	0.19 - 0.36	46479	0.0499	0.08697
GP36	0.42(0.09)	0.38 - 0.47	0.44(0.11)	0.40 - 0.51	43969	2.00E-04	**0.00072****
GP37	0.51(0.21)	0.41 - 0.62	0.48(0.21)	0.38 - 0.59	49121	0.0947	0.15031
GP38	0.89(0.24)	0.77 - 1.01	0.89(0.26)	0.77 - 1.03	48350	0.5516	0.6161
GP39	0.49(0.24)	0.42 - 0.66	0.52(0.26)	0.42 - 0.68	47849	0.3365	0.42764

Data presented as median interquartile range (IQR). Tests of significance were two tailed (p <0.05); q<0.05 significant after correction for FDR and are bold. W-Wilcoxon statistic

From **Table 6.2b,** low branching (LB), non-sialylated (S0), monogalactosylated (G1), core fucosylated (FUC_C), biantennary (BA) and biantennary galactosylated (A2G) glycans were higher among the controls compared to T2DM whereas high branching (HB), di-sialylated (S2), tri-sialylated (S3), trigalactosylated (G3), antennary

fucosylated (FUC_A), and triantennary (TRIA) were higher among T2DM individuals compared to controls (q<0.05).

Table 6.2b. Distribution of derived plasma N-glycan trait levels among cases and controls

	Control		Case				
N-glycan	Median(IQR)	Range	Median(IQR)	Range	W	p	q
Branching							
LB	82.65(3.60)	80.76 - 84.37	81.74(3.87)	79.66 - 83.53	46328	2.00E-04	**0.00072****
HB	16.94(3.84)	15.01 - 18.87	17.64(3.94)	16.00 - 19.95	44076	2.00E-04	**0.00072****
Level of sialylation							
S0	25.04(5.92)	22.02 - 27.94	23.63(5.30)	20.94 - 26.25	46929	0.0011	**0.00292****
S1	20.60(2.05)	19.60 - 21.65	20.46(2.11)	19.54 - 21.65	49856	0.2565	0.3477
S2	39.75(4.12)	37.52 - 41.64	40.74(4.25)	38.39 - 42.65	45743	0.0125	**0.02542****
S3	11.43(2.60)	9.92 - 12.53	11.91(2.70)	10.75 - 13.46	44101	2.00E-04	**0.00072****
S4	2.37(0.60)	2.07 - 2.67	2.41(0.68)	2.09 - 2.78	48044	0.413	0.51414
Level of galactosylation							
G0	9.74(3.17)	8.25 - 11.42	9.89(3.39)	8.24 - 11.64	48286	0.5209	0.6161
G1	10.64(2.61)	9.31 - 11.92	9.49(2.36)	8.41 - 10.78	44091	1.00E-05	**7.63E-05****
G2	62.06(5.17)	59.47 - 64.65	62.03(4.71)	59.57 - 64.29	50395	0.4585	0.55937
G3	12.24(3.42)	10.43 - 13.85	13.23(3.90)	10.99 - 14.90	44241	3.00E-04	**0.00096****
G4	4.45(1.52)	3.83 - 5.36	4.72(1.77)	3.89 - 5.67	47598	0.2525	0.3477
A2	8.78(3.28)	7.17 - 10.45	8.95(3.32)	7.29 - 10.62	48358	0.5555	0.6161
A2G	71.56(4.45)	69.35 - 73.8	70.25(4.01)	68.48 - 72.49	45663	1.00E-05	**7.63E-05****
Position of fucose							
FUC_A	2.10(0.60)	1.82 - 2.42	2.37(0.75)	1.98 - 2.74	43055	1.00E-05	**7.63E-05****
FUC_C	38.78(6.63)	35.20 - 41.84	36.57(6.69)	33.67 - 40.36	46497	3.00E-04	**0.00096****
Level of sialylation of biantennary glycans							
BAMS	20.60(2.05)	19.60 - 21.65	20.46(2.11)	19.54 - 21.65	49856	0.2565	0.3477
BADS	35.56(3.76)	33.57 - 37.32	36.06(3.82)	34.02 - 37.84	46886	0.0961	0.15031
Degree of branching							
BA	80.46(3.65)	78.61 - 82.27	79.67(3.94)	77.59 - 81.54	46376	2.00E-04	**0.00072****
TRIA	13.11 (3.47)	11.22 - 14.69	14.06(3.89)	11.90 - 15.8	44624	9.00E-04	**0.0025****
TA	4.74(1.62)	4.02 - 5.65	4.94(1.92)	4.05 - 5.97	47501	0.2243	0.3323

Data presented as median interquartile range (IQR). Tests of significance were two tailed (*p <0.05); **q<0.05 significant after correction for FDR and are bold. W-Wilcoxon statistic

Plasma N-glycan associations with T2DM

After performing logistic regression and FDR correction, GP4(FA2[6]G1), GP5 (FA2[3]G1), GP10 (FA2G2), GP13 (FA2[3]G1S[3]1), GP14 (A2G2S[6]1), GP16 (FA2G2S[6]1), GP17 (FA2BG2S[3]1), GP24 (A3G3S[3,6]2), GP 26 (A3G3S[3,3]2), GP32 (A3F1G3S[3,3,3]3) and GP36 (A4G4S[3,3,3,3]4) were significant in both models (q<0.05) **(Table 6.3a)**. Here, covariates were entered without forward or backward selection.

Table 6.3a. Logistic regression analysis of N-glycans in the baseline and full models

	BASELINE ADJUSTED					FULLY ADJUSTED				
	β	**S.E**	**OR(95%CI)**	**p**	**q**	**β**	**S.E**	**OR(95%CI)**	**p**	**q**
GP1	0.047	0.097	1.05(0.87 - 1.27)	0.63052	0.714444	-0.028	0.126	0.97(0.76 - 1.25)	0.82401	0.8707
GP2	-0.017	0.098	0.98(0.81 - 1.19)	0.8639	0.86487	0.099	0.132	1.10(0.85 - 1.43)	0.45234	0.6554
GP3	-0.25	0.100	0.78(0.64 - 0.95)	0.0120*	**0.024828****	-0.147	0.134	0.86(0.66 - 1.12)	0.27148	0.4791
GP4	-0.544	0.107	0.58(0.47 - 0.72)	0.00001*	**6.67E-05****	-0.496	0.133	0.61(0.47 - 0.79)	0.0002	**0.0042****
GP5	-0.486	0.104	0.62(0.50 - 0.75)	0.00001*	**6.67E-05****	-0.5	0.133	0.61(0.47 - 0.79)	0.00018	**0.0042****
GP6	-0.276	0.096	0.76(0.63 - 0.92)	0.00394*	**0.009092****	-0.188	0.124	0.83(0.65 - 1.06)	0.12893	0.2658
GP7	0.043	0.098	1.04(0.86 - 1.27)	0.66105	0.721145	0.069	0.127	1.07(0.84 - 1.38)	0.58538	0.7317
GP8	-0.072	0.101	0.93(0.76 - 1.13)	0.47517	0.595102	0.211	0.14	1.24(0.94 - 1.63)	0.1329	0.2658
GP9	-0.158	0.098	0.85(0.70 - 1.04)	0.1072	0.173838	-0.097	0.136	0.91(0.70 - 1.19)	0.47656	0.6554
GP10	-0.523	0.111	0.59(0.48 - 0.74)	0.00001*	**6.67E-05****	-0.368	0.141	0.69(0.53 - 0.91)	0.00934	**0.0313****
GP11	-0.351	0.101	0.70(0.58 - 0.86)	0.00051*	**0.001457****	-0.256	0.132	0.77(0.60 - 1.00)	0.05184	0.1152
GP12	0.098	0.098	1.10(0.91 - 1.34)	0.31796	0.433582	0.078	0.132	1.08(0.83 - 1.40)	0.55429	0.7076
GP13	-0.237	0.095	0.79(0.65 - 0.95)	0.0129*	**0.0258****	-0.312	0.125	0.73(0.57 - 0.94)	0.01243	**0.0373****
GP14	0.258	0.097	1.29(1.07 - 1.57)	0.0081*	**0.017357****	0.372	0.13	1.45(1.12 - 1.87)	0.00433	**0.0278****
GP15	0.039	0.095	1.04(0.86 - 1.25)	0.6848	0.733714	-0.005	0.127	1.00(0.78 - 1.28)	0.96892	0.9853
GP16	-0.385	0.106	0.68(0.55 - 0.84)	0.00027*	**0.000853****	-0.335	0.133	0.72(0.55 - 0.93)	0.01159	**0.0366****
GP17	-0.344	0.100	0.71(0.58 - 0.86)	0.00059*	**0.001609****	-0.341	0.131	0.71(0.55 - 0.92)	0.00939	**0.0313****
GP18	-0.283	0.101	0.75(0.62 - 0.92)	0.00524*	**0.011644****	-0.066	0.134	0.94(0.72 - 1.22)	0.6233	0.732
GP19	-0.043	0.092	0.96(0.80 - 1.15)	0.64299	0.714444	-0.09	0.127	0.91(0.71 - 1.17)	0.48066	0.6554
GP20	0.183	0.096	1.20(1.00 - 1.45)	0.05672	0.103127	0.18	0.126	1.20(0.94 - 1.53)	0.15165	0.2935
GP21	-0.128	0.099	0.88(0.73 - 1.07)	0.19371	0.270293	-0.094	0.13	0.91(0.71 - 1.18)	0.47074	0.6554
GP22	0.192	0.096	1.21(1.00 - 1.46)	0.04558*	0.085463	-0.052	0.123	0.95(0.75 - 1.21)	0.67356	0.7625
GP23	-0.173	0.097	0.84(0.70 - 1.02)	0.07371	0.130076	-0.277	0.125	0.76(0.59 - 0.97)	0.02597	0.0677
GP24	0.321	0.105	1.38(1.12 - 1.69)	0.00228*	**0.005472****	0.388	0.14	1.48(1.12 - 1.94)	0.00556	**0.0278****
GP25	0.171	0.099	1.19(0.98 - 1.44)	0.08286	0.142046	0.112	0.132	1.12(0.86 - 1.45)	0.39576	0.6475
GP26	0.636	0.120	1.89(1.49 - 2.39)	0.00001*	**6.67E-05****	0.548	0.155	1.73(1.28 - 2.34)	0.00041	**0.0062****
GP27	0.017	0.098	1.02(0.84 - 1.23)	0.86487	0.86487	0.061	0.129	1.06(0.83 - 1.37)	0.63444	0.732
GP28	0.15	0.099	1.16(0.96 - 1.41)	0.13001	0.205279	0.156	0.133	1.17(0.90 - 1.52)	0.23924	0.435
GP29	-0.406	0.104	0.67(0.54 - 0.82)	0.0001*	**0.000353****	-0.255	0.132	0.78(0.60 - 1.01)	0.05436	0.1165
GP30	0.343	0.108	1.41(1.14 - 1.74)	0.00146*	**0.00365****	0.304	0.142	1.36(1.03 - 1.79)	0.03189	0.0797
GP31	0.435	0.112	1.55(1.24 - 1.92)	0.0001*	**0.000353****	0.298	0.148	1.35(1.01 - 1.80)	0.04491	0.1078
GP32	0.635	0.117	1.89(1.50 - 2.38)	0.00001*	**6.67E-05****	0.472	0.156	1.60(1.18 - 2.18)	0.0025	**0.0278****
GP33	0.02	0.097	1.02(0.84 - 1.24)	0.83359	0.862334	0.084	0.129	1.09(0.85 - 1.40)	0.51431	0.6708
GP34	0.568	0.120	1.76(1.40 - 2.23)	0.00001*	**6.67E-05****	0.362	0.16	1.44(1.05 - 1.97)	0.0234	0.0638
GP35	0.164	0.097	1.18(0.97 - 1.42)	0.09083	0.151383	0.106	0.127	1.11(0.87 - 1.43)	0.40378	0.6475
GP36	0.396	0.106	1.49(1.21 - 1.83)	0.00019*	**0.000633****	0.359	0.136	1.43(1.10 - 1.87)	0.00831	**0.0312****
GP37	-0.135	0.099	0.87(0.72 - 1.06)	0.17052	0.2436	-0.038	0.13	0.96(0.75 - 1.24)	0.7734	0.8437
GP38	0.067	0.100	1.07(0.88 - 1.30)	0.49840	0.59808	0.102	0.133	1.11(0.85 - 1.44)	0.4446	0.6554
GP39	0.047	0.095	1.05(0.87 - 1.26)	0.62330	0.714444	0.045	0.122	1.05(0.82 - 1.33)	0.71499	0.7944

Baseline model: Age and gender adjusted. Full model: Age, gender, BMI, TC, TG, FPG and LDL-c. Tests of significance were two tailed (*p <0.05); **q<0.05 significant after correction for FDR and are bold.

After performing logistic regression and FDR correction, LB, HB, S3, G1, G3, FUC_C, BA, A2G and TRIA were significant in both models ($p<0.05;q<0.05$), while S0 and FUC_A were significant in the fully adjusted model, but not in baseline adjusted **(Table 6.3b)**.

Table 6.3b Regression analyses of derived N-glycans for baseline adjusted and fully adjusted.

	BASELINE ADJUSTED					FULLY ADJUSTED				
Glycan	**β**	**S.E**	**OR(95%CI)**	**p**	**q**	**β**	**S.E.**	**OR(95%CI)**	**p**	**q**
LB	-0.37	0.136	0.69(0.53 - 0.90)	0.00656*	**0.0281****	-0.446	0.109	0.64(0.52 - 0.79)	0.00004*	**0.000171****
HB	0.403	0.142	1.50(1.13 - 1.98)	0.00448*	**0.0278****	0.46	0.11	1.59(1.28 - 1.97)	0.00003*	**0.000138****
S0	-0.251	0.127	0.78(0.61 - 1.00)	0.04893*	0.1129	-0.32	0.101	0.73(0.60 - 0.88)	0.00145*	**0.00365****
S1	-0.062	0.127	0.94(0.73 - 1.20)	0.62346	0.732	-0.144	0.097	0.87(0.72 - 1.05)	0.13772	0.20658
S2	0.161	0.126	1.18(0.92 - 1.51)	0.20087	0.3766	0.219	0.098	1.25(1.03 - 1.51)	0.02598*	0.050284
S3	0.371	0.139	1.45(1.10 - 1.90)	0.00773*	**0.0309****	0.457	0.109	1.58(1.28 - 1.95)	0.00003*	**0.000138****
S4	0.104	0.129	1.11(0.86 - 1.43)	0.42085	0.6475	0.068	0.097	1.07(0.88 - 1.30)	0.486	0.595102
G0	0.017	0.128	1.02(0.79 - 1.31)	0.89658	0.9275	0.045	0.098	1.05(0.86 - 1.27)	0.643	0.714444
G1	-0.49	0.132	0.61(0.47 - 0.79)	0.00021*	**0.0042****	-0.52	0.104	0.60(0.49 - 0.73)	0.00001*	**6.67E-05****
G2	-0.027	0.124	0.97(0.76 - 1.24)	0.82715	0.8707	-0.094	0.096	0.91(0.75 - 1.10)	0.33002	0.440027
G3	0.422	0.147	1.53(1.14 - 2.04)	0.00412*	**0.0278****	0.484	0.113	1.62(1.30 - 2.03)	0.00002*	**0.00012****
G4	0.102	0.124	1.11(0.87 - 1.41)	0.41174	0.6475	0.068	0.095	1.07(0.89 - 1.29)	0.47749	0.595102
FUC_A	0.315	0.134	1.37(1.05 - 1.78)	0.01871*	0.0535	0.461	0.105	1.59(1.29 - 1.95)	0.00001*	**6.67E-05****
FUC_C	-0.363	0.13	0.70(0.54 - 0.90)	0.00511*	**0.0278****	-0.352	0.101	0.70(0.58 - 0.86)	0.00048*	**0.00144****
BA	-0.385	0.138	0.68(0.52 - 0.89)	0.00516*	**0.0278****	-0.451	0.109	0.64(0.52 - 0.79)	0.00003*	**0.000138****
A2	-0.001	0.128	1.00(0.78 - 1.28)	0.99191	0.9919	0.036	0.098	1.04(0.86 - 1.26)	0.70985	0.747211
A2G	-0.382	0.136	0.68(0.52 - 0.89)	0.00495*	**0.0278****	-0.48	0.107	0.62(0.50 - 0.76)	0.00001*	**6.67E-05****
BAMS	-0.062	0.127	0.94(0.73 - 1.20)	0.62346	0.732	-0.144	0.097	0.87(0.72 - 1.05)	0.13772	0.20658
BADS	0.083	0.125	1.09(0.85 - 1.39)	0.50657	0.6708	0.137	0.097	1.15(0.95 - 1.39)	0.15766	0.230722
TRIA	0.396	0.145	1.49(1.12 - 1.97)	0.00633*	**0.0281****	0.451	0.112	1.57(1.26 - 1.95)	0.00005*	**0.0002****
TA	0.102	0.124	1.11(0.87 - 1.41)	0.40823	0.6475	0.075	0.095	1.08(0.90 - 1.30)	0.42856	0.558991

Baseline model: Age and gender adjusted. Full model: Age, gender, BMI, FPG, TC, TG and LDL-c. Tests of significance were two tailed (*p <0.05); **q<0.05 significant after correction for FDR and are bold.

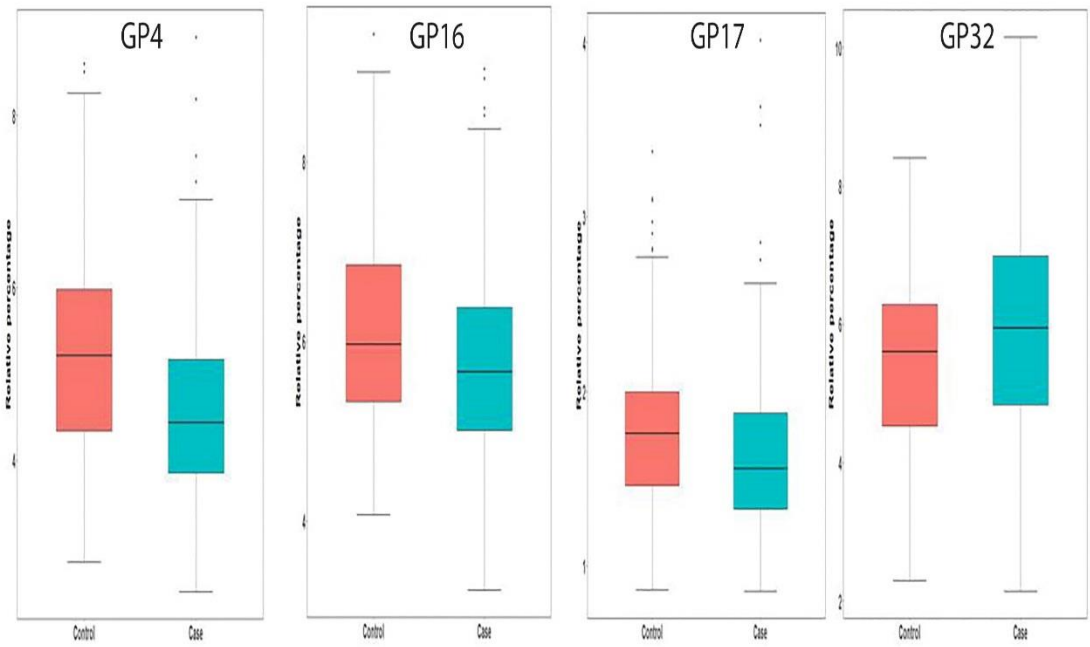

Figure 6.2. Differences in expression of selected N-glycans after performing a logistic regression in the full model. While GP4 - FA2[6]G1, GP16 - FA2G2S[6]1 and GP17 - FA2BG2S[3]1 are higher in controls, GP32 -A3F1G3S[3,3,3]3 is higher in T2DM.

After controlling for FDR, 9 N-glycan traits correlated with WHtR among the controls (6 positively and 3 negatively). Among the cases, 6 N-glycan traits correlated with WHtR (2 positively and 4 negatively). There were 13 correlations between N-glycans and LDL-c among the controls (7 positively and 6 negatively). However, no correlations were found between N-glycan traits and LDL-c in the cases. Whereas there were 13 correlations between N-glycans and TC among the controls (7 positively and 6 negatively), there were no correlations between derived N-glycan traits and TC in T2DM.

Further, 13 significant correlations were found between derived N-glycan traits and TG in controls (7 positively and 6 negatively) whereas 4 positive correlations were shown between derived plasma N-glycan traits and TG among the cases **(Table 6.4)**.

Table 6.4. Correlations between derived plasma N-glycan traits and WHtR, FPG, LDL-c, TC and TG

	WHtR				**FPG**				**LDL-c**				**TC**				**TG**			
	CONTROLS		CASES		CONTROLS		CASES		CONTROLS		CASES		CONTROLS		CASES		CONTROLS		CASES	
Peaks	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q
Branching																				
LB	-0.22	**0.0025****	-0.15	0.0568	-0.02	0.862	-0.13	0.2704	-0.21	**0.0029****	0.03	0.9927	-0.26	**0.0001****	-0.02	0.9522	-0.23	**0.0006****	-0.14	0.0933
HB	0.25	**0.0005****	0.15	0.0599	0.02	0.862	0.12	0.3084	0.21	**0.0032****	-0.01	0.9927	0.26	**0.0001****	0.04	0.9522	0.25	**0.0006****	0.15	0.0768
Level of sialylation																				
S0	-0.14	0.0599	-0.01	0.9618	-0.06	0.5835	-0.11	0.3144	-0.20	**0.0035****	-0.02	0.9927	-0.20	**0.0040****	-0.06	0.9522	-0.13	**0.0078****	-0.11	0.2173
S1	-0.14	0.0599	-0.29	**0.0001****	-0.09	0.3677	0.07	0.5611	-0.04	0.5837	0.06	0.9927	-0.09	0.2268	0.03	0.9522	-0.17	0.3074	-0.06	0.5652
S2	0.15	0.054	0.07	0.4993	0.16	0.1454	0.04	0.8136	0.17	**0.013****	0.03	0.9927	0.17	**0.0165****	0.07	0.9522	0.13	**0.0296****	0.12	0.2042
S3	0.24	**0.0011****	0.15	0.0568	0.04	0.7486	0.11	0.3144	0.18	**0.0104****	-0.02	0.9927	0.24	**0.0006****	0.03	0.9522	0.24	**0.0018****	0.14	0.1009
S4	0.18	**0.0205****	0.08	0.4094	-0.14	0.1659	0.06	0.5938	0.22	**0.0019****	-0.03	0.9927	0.21	**0.0022****	0.00	0.9917	0.16	**0.0053****	0.11	0.2173
Level of galactosylation																				
G0	-0.08	0.3266	0.03	0.801	0.03	0.7677	-0.03	0.8388	-0.04	0.5854	-0.01	0.9927	-0.01	0.8793	0.00	0.9917	0.01	0.9248	0.03	0.7909
G1	-0.11	0.1495	0.01	0.9618	-0.1	0.3554	-0.11	0.3144	-0.26	**0.0002****	-0.02	0.9927	-0.24	**0.0005****	-0.08	0.9522	-0.15	**0.0016****	-0.15	0.0768
G2	-0.05	0.5756	-0.16	0.0521	0.04	0.7486	0.00	0.99	0.02	0.7833	0.05	0.9927	-0.04	0.5276	0.02	0.9522	-0.09	0.6072	-0.05	0.5652
G3	0.29	**0.0001****	0.15	0.0568	0.01	0.862	0.13	0.2704	0.19	**0.0054****	0.00	0.9927	0.28	**0.0001****	0.06	0.9522	0.25	**0.0004****	0.18	**0.0492****
G4	0.00	0.9767	0.01	0.9651	0.01	0.8627	-0.02	0.8388	0.07	0.3072	0.00	0.9927	0.05	0.4907	0.01	0.9522	0.06	0.5757	-0.02	0.8382
A2	-0.09	0.2679	0.04	0.7686	0.03	0.7677	-0.04	0.8233	-0.05	0.4799	-0.02	0.9927	-0.02	0.7559	0.00	0.9917	0.01	0.8089	0.02	0.8379
A2G	-0.16	**0.0362****	-0.18	**0.0257****	-0.04	0.7486	-0.08	0.4996	-0.17	**0.0181****	0.04	0.9927	-0.23	**0.0012****	-0.02	0.9522	-0.21	**0.0034****	-0.14	0.11
Position of fucose																				
FUC_A	0.33	**0.0001****	0.27	**0.0002****	0.15	0.1517	0.10	0.3292	0.16	**0.0195****	-0.02	0.9927	0.20	**0.0037****	0.03	0.9522	0.31	**0.0078****	0.22	**0.0106****
FUC_C	-0.12	0.1251	0.01	0.9618	-0.08	0.4805	-0.05	0.7009	-0.24	**0.0007****	0.00	0.9927	-0.23	**0.0010****	-0.05	0.9522	-0.16	**0.0028****	-0.11	0.2173
Level of sialylation of biantennary *N*-glycans																				
BAMS	-0.14	0.0599	-0.29	**0.0001****	-0.09	0.3677	0.07	0.5611	-0.04	0.5837	0.06	0.9927	-0.09	0.2268	0.03	0.9522	-0.17	0.3074	-0.06	0.5652
BADS	0.09	0.2679	0.04	0.7686	0.14	0.1659	0.02	0.8643	0.12	0.0824	0.03	0.9927	0.10	0.1466	0.06	0.9522	0.07	0.2181	0.08	0.40
Degree of branching																				
BA	-0.25	**0.0005****	-0.16	**0.0495****	-0.03	0.823	-0.14	0.2704	-0.24	**0.0006****	0.01	0.9927	-0.30	**0.0000****	-0.04	0.9522	-0.26	**0.0002****	-0.16	0.0768
TRIA	0.31	**0.0001****	0.16	**0.0495****	0.02	0.8272	0.13	0.2704	0.21	**0.0027****	0.01	0.9927	0.29	**0.0000****	0.06	0.9522	0.26	**0.0002****	0.18	**0.0492****
TA	0.00	0.9767	0.01	0.9651	0.02	0.8272	-0.02	0.8388	0.07	0.2913	0.00	0.9927	0.05	0.4624	0.02	0.9522	0.07	0.5531	-0.02	0.8382

Tests of significance were two tailed **q<0.05 significant after correction for FDR and are bold.

As shown in **Table 6.5a**, 19 N-glycan peaks correlated with WHtR among the controls (10 positively and 9 negatively) whereas 12 N-glycan peaks correlated with WHtR among the cases (7 positively and 5 negatively). No significant correlations existed between N-glycan peaks and FPG in both controls and cases. There were 22 correlations between N-glycan peaks and TC among the controls (13 positively and 9 negatively) whereas no correlations were seen among the cases.

Table 6.5a. Correlation between plasma N-glycan peaks and WHtR, FPG, TC and TG

	WHtR				FPG				TC			
	controls		Cases		Control		Case		Controls		Cases	
Peak	rs	q	rs	q	rs	q	rs	q	rs	q	rs	q
GP1	-0.06	0.4575	0.08	0.4114	0.04	0.7486	-0.01	0.884	-0.02	0.8045	0.00	0.9917
GP2	-0.19	**0.0113****	-0.08	0.4094	0.00	0.9841	-0.10	0.3685	0.00	0.9897	-0.03	0.9522
GP3	-0.29	**0.0001****	-0.21	**0.0068****	-0.14	0.1659	-0.07	0.4996	-0.27	**0.0004****	-0.05	0.9522
GP4	-0.12	0.1251	0.03	0.801	-0.08	0.4805	-0.11	0.3144	-0.22	**0.0044****	-0.08	0.9522
GP5	-0.06	0.4478	0.04	0.7489	-0.11	0.3106	-0.10	0.3302	-0.26	**0.0007****	-0.10	0.9522
GP6	-0.02	0.8185	0.11	0.1745	-0.04	0.7628	-0.13	0.2704	-0.09	0.3074	-0.09	0.9522
GP7	0.17	**0.0305****	0.00	0.9652	-0.04	0.7486	0.02	0.8388	0.17	**0.0281****	-0.02	0.9522
GP8	-0.32	**0.0001****	-0.33	**0.0001****	-0.12	0.3064	-0.11	0.3144	-0.21	**0.0054****	-0.02	0.9522
GP9	-0.26	**0.0003****	-0.21	**0.0064****	-0.16	0.1454	0.02	0.8388	-0.21	**0.0061****	-0.04	0.9522
GP10	-0.18	**0.0205****	-0.09	0.3481	-0.17	0.1454	-0.14	0.2704	-0.28	**0.0003****	-0.11	0.9522
GP11	-0.05	0.5756	-0.04	0.7686	-0.11	0.3106	-0.03	0.8388	-0.09	0.2646	-0.11	0.9522
GP12	-0.15	0.054	-0.31	**0.0001****	0.04	0.7486	0.20	0.1568	0.21	**0.0053****	0.08	0.9522
GP13	-0.10	0.2254	0.03	0.8537	-0.15	0.1454	-0.04	0.8136	-0.25	**0.0014****	-0.03	0.9522
GP14	-0.01	0.9372	-0.22	**0.0039****	0.10	0.3677	0.00	0.9900	0.14	0.0691	0.06	0.9522
GP15	-0.09	0.2679	-0.11	0.2187	-0.14	0.1659	0.14	0.2704	-0.13	0.1024	0.05	0.9522
GP16	-0.18	**0.0169****	-0.14	0.0721	-0.17	0.1454	0.00	0.9900	-0.24	**0.0016****	-0.05	0.9522
GP17	0.05	0.5032	-0.01	0.9618	-0.07	0.5281	0.04	0.8142	0.02	0.8032	-0.06	0.9522
GP18	-0.37	**0.0001****	-0.33	**0.0001****	-0.11	0.3106	-0.11	0.3144	0.03	0.7459	-0.05	0.9522
GP19	0.32	**0.0001****	0.27	**0.0002****	0.17	0.1454	0.04	0.8136	0.35	**0.0001****	0.13	0.9522
GP20	0.15	0.054	0.08	0.4105	0.15	0.1454	-0.02	0.8643	0.15	0.0603	0.05	0.9522
GP21	-0.08	0.2954	-0.05	0.6698	-0.02	0.8314	-0.01	0.9421	-0.20	**0.0078****	0.02	0.9522
GP22	0.06	0.4575	0.04	0.7686	0.08	0.5008	0.16	0.2704	-0.07	0.3939	0.02	0.9522
GP23	-0.02	0.8185	0.01	0.9651	-0.05	0.7486	0.07	0.4996	-0.11	0.1642	0.00	0.9917
GP24	0.09	0.2407	-0.01	0.9651	-0.05	0.7229	0.06	0.6218	0.19	**0.0150****	0.03	0.9522
GP25	-0.17	**0.0271****	-0.17	**0.0433****	-0.07	0.5165	0.02	0.851	0.08	0.3181	0.01	0.9917
GP26	0.38	**0.0001****	0.22	**0.0051****	0.08	0.5008	0.13	0.2704	0.30	**0.0001****	0.07	0.9522
GP27	-0.16	**0.0375****	-0.06	0.5571	0.06	0.6619	-0.09	0.3866	-0.06	0.5033	0.02	0.9522
GP28	0.04	0.5756	-0.01	0.9618	-0.10	0.3554	0.07	0.4996	0.20	**0.0078****	0.02	0.9522
GP29	-0.28	**0.0001****	-0.15	0.0568	-0.16	0.1454	-0.05	0.6611	0.00	0.994	0.02	0.9522
GP30	0.26	**0.0004****	0.13	0.1246	-0.03	0.7628	0.12	0.3084	0.25	**0.0014****	0.04	0.9522
GP31	0.37	**0.0001****	0.21	**0.0057****	0.07	0.5181	0.14	0.2704	0.36	**0.0001****	0.06	0.9522
GP32	0.49	**0.0001****	0.33	**0.0001****	0.14	0.1659	0.16	0.2704	0.28	**0.0003****	0.07	0.9522
GP33	-0.15	0.0529	-0.06	0.6092	0.04	0.7486	-0.09	0.4241	-0.06	0.4729	0.01	0.9917
GP34	0.44	**0.0001****	0.29	**0.0001****	0.10	0.3677	0.16	0.2704	0.37	**0.0001****	0.05	0.9522
GP35	-0.02	0.8185	0.05	0.6698	0.11	0.3106	-0.03	0.8388	0.06	0.5281	0.02	0.9522
GP36	0.27	**0.0002****	0.10	0.2700	-0.01	0.862	0.11	0.3144	0.32	**0.0001****	0.04	0.9522
GP37	0.09	0.2467	0.00	0.9652	-0.19	0.1434	0.02	0.8388	0.14	0.0797	0.00	0.9917
GP38	0.26	**0.0004****	0.12	0.1593	-0.15	0.1517	0.09	0.3889	0.27	**0.0004****	0.05	0.9522
GP39	0.02	0.8185	0.07	0.5233	0.05	0.7229	-0.02	0.8388	0.01	0.9637	0.03	0.9522

Tests of significance were two tailed **q<0.05 significant after correction for FDR and are bold.

As shown in **Table 6.5b**, 14 N-glycan peaks correlated with TG among the controls while 5 correlated with TG among the cases. There were 19 correlations between GPs and LDL-c among controls whereas no correlations were seen in the cases.

Table 6.5b. Correlation between plasma N-glycan peaks, TG, and LDL-c.

	TG				LDL-c			
	Controls		**Cases**		Controls		Cases	
Peak	rs	q	rs	q	rs	q	rs	q
GP1	0.01	0.9087	0.02	0.8379	-0.06	0.4791	-0.02	0.9927
GP2	-0.05	0.5469	0.02	0.8379	0.01	0.875	-0.02	0.9927
GP3	-0.14	0.0829	-0.02	0.8379	-0.27	**0.0013****	-0.01	0.9927
GP4	-0.14	0.0992	-0.17	0.0658	-0.23	**0.0037****	-0.03	0.9927
GP5	-0.11	0.169	-0.17	0.0658	-0.29	**0.0006****	-0.05	0.9927
GP6	-0.10	0.2697	-0.02	0.8379	-0.09	0.2836	-0.07	0.9927
GP7	0.11	0.1986	0.07	0.4913	0.15	0.0599	-0.04	0.9927
GP8	-0.15	0.0808	-0.05	0.5652	-0.15	0.0587	0.03	0.9927
GP9	-0.18	**0.0317****	-0.03	0.8163	-0.20	**0.0132****	-0.01	0.9927
GP10	-0.22	**0.0057****	-0.26	**0.0020****	-0.25	**0.0024****	-0.05	0.9927
GP11	-0.17	**0.0445****	-0.08	0.4277	-0.09	0.2836	-0.10	0.9927
GP12	-0.04	0.6813	0.06	0.5652	0.24	**0.0027****	0.05	0.9927
GP13	-0.14	0.0992	-0.11	0.2173	-0.25	**0.0016****	0.02	0.9927
GP14	0.05	0.5325	0.04	0.6962	0.19	**0.0186****	0.05	0.9927
GP15	-0.09	0.3124	0.08	0.3874	-0.11	0.1609	0.04	0.9927
GP16	-0.21	**0.0074****	-0.17	0.0599	-0.20	**0.0112****	0.01	0.9927
GP17	-0.10	0.2389	0.00	0.9987	0.00	0.9532	-0.08	0.9927
GP18	-0.22	**0.0074****	-0.17	0.0599	0.09	0.3028	-0.04	0.9927
GP19	0.34	**0.0009****	0.15	0.0826	0.35	**0.0006****	0.13	0.9927
GP20	0.12	0.1489	0.11	0.2173	0.17	**0.0351****	0.01	0.9927
GP21	-0.01	0.9087	0.07	0.4876	-0.19	**0.0132****	0.02	0.9927
GP22	0.05	0.5325	0.05	0.5652	-0.09	0.301	0.03	0.9927
GP23	-0.07	0.4025	-0.01	0.9249	-0.12	0.1583	0.02	0.9927
GP24	0.07	0.4366	0.08	0.4277	0.14	0.0741	0.00	0.9927
GP25	-0.12	0.169	-0.08	0.418	0.04	0.6197	0.01	0.9927
GP26	0.29	**0.0006****	0.20	**0.0196****	0.21	**0.0093****	0.03	0.9927
GP27	-0.04	0.6813	-0.12	0.181	-0.03	0.7272	0.04	0.9927
GP28	0.06	0.5289	0.06	0.5652	0.15	0.0587	-0.01	0.9927
GP29	-0.20	**0.0124****	-0.11	0.2173	0.07	0.3854	0.02	0.9927
GP30	0.21	**0.0078****	0.15	0.0768	0.17	**0.036****	0.00	0.9927
GP31	0.31	**0.0002****	0.26	**0.002****	0.26	**0.0013****	-0.01	0.9927
GP32	0.37	**0.0009****	0.28	**0.0006****	0.20	**0.0114****	0.01	0.9927
GP33	-0.03	0.7552	-0.11	0.2173	-0.03	0.7272	0.02	0.9927
GP34	0.39	**0.0009****	0.30	**0.0006****	0.27	**0.0013****	-0.02	0.9927
GP35	0.07	0.4366	-0.02	0.8379	0.06	0.462	0.00	0.9927
GP36	0.24	**0.0023****	0.15	0.0768	0.29	**0.0006****	-0.01	0.9927
GP37	0.03	0.7035	0.05	0.5705	0.15	**0.0587****	-0.01	0.9927
GP38	0.21	**0.0092****	0.15	0.0768	0.27	**0.0013****	0.00	0.9927
GP39	0.08	0.3599	0.05	0.5652	0.03	0.7272	0.00	0.9927

Tests of significance were two tailed **q<0.05 significant after correction for FDR and are bold.

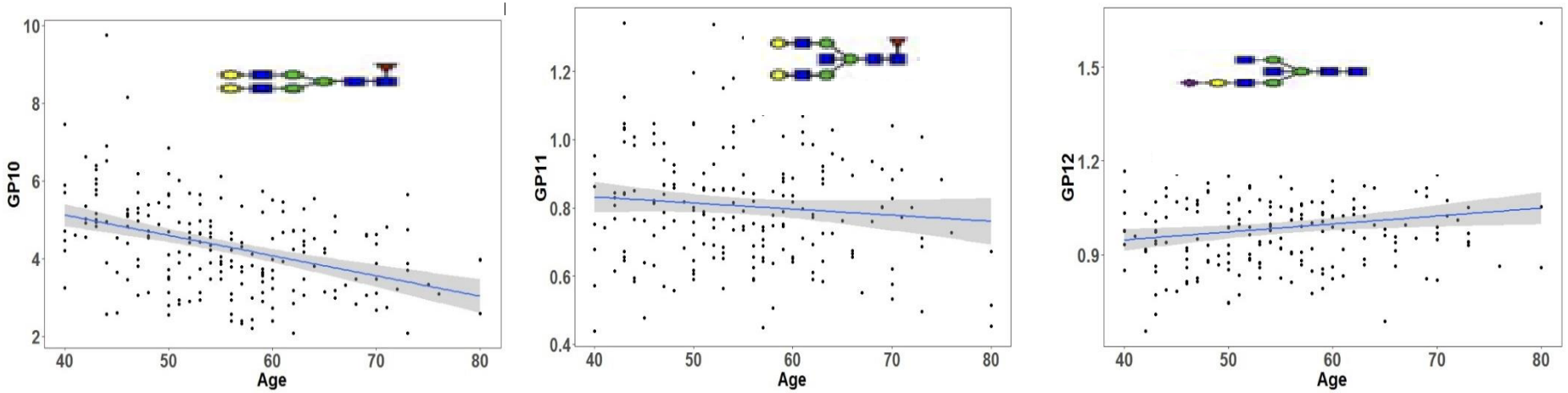

Figure 6.3a. Line graph of the relationship between selected N-glycans and age in T2DM. Overall, advancing in age was associated with decreasing levels of GP10 (FA2G2) and GP11 (FA2BG2) whereas increasing in age was associated with increasing levels of GP12 (A2[3] BG1S[3]1).

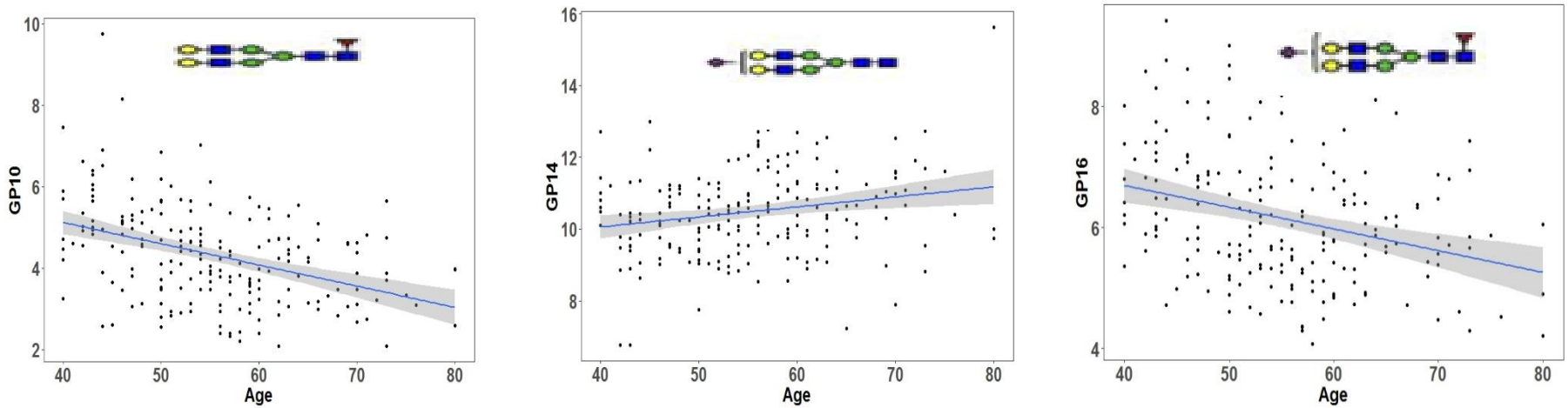

Figure 6.3b. A line graph of the relationship between selected N-glycans and Age among controls. The shaded region represents 95% confidences interval on the fitted values.Advancing in age was associated with decreasing levels of GP10 (FA2G2) and GP16 (FA2G2S [6]1) whereas increasing in age was associated with increasing levels of GP14(A2G26 [6]1).

Table 6.6 Adjusted logistic regression model

			Model Fitting Criteria				
	Beta	S.E	AIC	BIC	-2 Log Likelihood	$\chi 2$	q
GP1	0.342	0.073	521.311	558.255	503.311	27.584	**0.000**
GP4	-1.109	0.16	554.319	591.262	536.319	60.592	**0.000**
GP7	5.639	1.088	524.123	561.066	506.123	30.396	**0.000**
GP11	4.381	1.538	502.132	539.075	484.132	8.405	**0.004**
GP17	-3.199	0.57	529.583	566.526	511.583	35.856	**0.000**
GP19	-4.421	0.914	519.778	556.721	501.778	26.051	**0.000**
GP22	0.849	0.171	521.073	558.016	503.073	27.346	**0.000**
GP26	1.496	0.428	506.602	543.545	488.602	12.875	**0.000**
GP29	-6.113	2.951	498.112	535.056	480.112	4.385	**0.036**

S.E Standard error of the beta. AIC: Akaike information criterion, BIC: Bayesian information criterion, $\chi 2$: Chi-square. Tests of statistical significance are two tailed and q<0.05 is bold

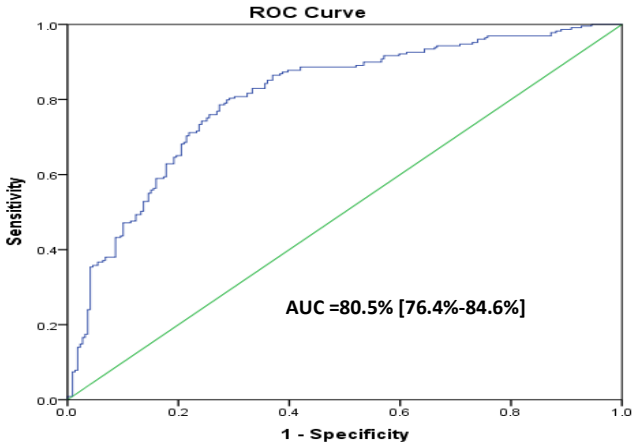

Figure 6.4. Receiver operative characteristic (ROC) curve illustrating the performance of the age and sex adjusted logistic regression model in predicting the status of patients with T2DM and healthy controls. Nine GPs were found to predict case status using a step-wise Akaike's information criterion (AIC) and Bayesian information criterion (BIC) logistic regression model selection (Table 6.6). This model yielded an area under curve of 80.5% [95% CI 76.4%-84.6%].

6.6 Discussion

This first comprehensive whole human plasma N-glycan profiling study using a Ghanaian population is premised on the hypothesis that there is a differential abundance of plasma N-glycan structures between T2DM and healthy controls.

Association between T2DM risk factors and measured plasma N-glycans

Several T2DM risk factors were significantly associated with either increased or decreased levels of specific plasma N-glycans **(Tables 6.4 & 6.5)**. Consistent with previous findings (Krištić et al., 2014; Lemmers et al., 2017; Perkovic et al., 2014; Reiding et al., 2017; Russell et al., 2017; Yu et al., 2016), the present study has shown that there were significant correlations between plasma N-glycans and age (**Figure 6.3**), WHtR, BMI and TG in both cases and controls whereas there were no correlations

between N-glycans and HDL-c in both groups. Surprisingly, nearly all significant correlations between N-glycans and TC, FPG, LDL-c, SBP and DBP **(Tables 6.4, 6.5a & 6.5b and Supplementary Tables S2-4, See Appendix)** were only observed among the controls. This outcome could partly be attributed to the impact of medications. All T2DM patients were recruited from a hospital and our previous investigations among these people revealed that they were on glucose lowering, lipid lowering and anti-hypertensive therapy at the time of blood sampling (Adua et al., 2017).

Core and antennary fucosylation

While it is still not well understood, altered plasma N-glycosylation patterns between T2DM and healthy controls could be attributed to the differential expression of glycosyltransferases particularly α-1,6-fucosyltransferase 8 (FUT8) which catalyses core fucosylation (Simala-Grant, & Taylor, 2006; Yarema & Bertozzi, 2001). Core fucosylation, the attachment of fucose to inner *N*-acetylglucosamine (GlcNAc), is a significant phenomenon in the glycosylation machinery that mediates important cellular events, including notch signalling, growth factor receptor expression and adhesion molecule activity. Thus, its alteration has been linked to many metabolic events such as inflammation and T2DM (Ma et al., 2006; Miyoshi, Moriwaki, & Nakagawa, 2008). For example, an increased core fucose level was associated with diabetes mellitus (DM) (McMillan, 1972) and α-1,6 core fucosylation was higher in the sera of db/db mice (a model of T2DM with obesity) and humans with T2DM (Itoh et al., 2007). On the contrary, our results show that T2DM patients had a decreased FUC_C (**Table 6.2b)** and additionally, core fucosylated N-glycans with bisection; GP5 (FA2[3]G1), GP11(FA2BG2) and GP17 (FA2BG2S[3]1) were significantly decreased in T2DM **(Table 6.2a)**. Our findings however, are consistent with recent N-glycomics investigations involving larger populations (Lemmers et al., 2017; McLachlan et al., 2016). For example, in a large cohort study comprising 2,155 individuals from the Orkney Islands (UK), a subclass of individuals suffering from metabolic syndrome (MetS) had decreased levels of FUC_C N-glycans (McLachlan et al., 2016). A possible explanation for this is that decreased expression of FUT8 or decreased/absence of fucosyltransferase may lead to overexpression of matrix metalloproteinase and decreased expression of extracellular matrix proteins. This inhibits the binding of epidermal growth factor (EGF) to its receptor in the transforming growth factor β1 signalling pathway. Also, absence of core fucosylation leads to impaired function of low-density lipoprotein receptor-related protein 1, which is involved in the endocytosis of insulin-like growth

factor (IGF)-binding protein-3 (IGFBP-3) (Brinkman-van der Linden, de Haan, Havenaar, & van Dijk, 1998; Ma et al., 2006). Alternatively, β-1,4 *N*-acetylglucosaminyltranferase III (GnT III) is an enzyme that catalyses the addition to *N*-acetylglucosamine to the β mannose of the core of the trimannosyl core of N-glycans but also suppresses the elongation or the formation of multiantennary chains on N-glycans (Li et al., 2007). Moreover, while the focus of this study was not on specific proteins, it is worth noting that the glycosylation of certain plasma proteins has implications for T2DM pathophysiology. For example, one study has shown that decreased FUC_C IgG N-glycans, with or without bisecting GIcNAc, was associated with T2DM in European populations (Lemmers et al., 2017). Particularly, bisecting *N*-acetyl glucosamine on IgG facilitates antibody dependent cellular cytotoxicity (ADCC) or a pro-inflammatory state since the presence of bisecting *N*-acetyl glucosamine on N-glycans inhibits core fucosylation and therefore indirectly promotes the binding of IgG molecules to Fcγ receptor III (Russell et al., 2018).

In some cases, fucose is attached to C-3 and C-4 of GlcNAc of an antennary glycan in which case it is referred to as antennary fucosylation (FUC_A). Similar to FUC_C, dysregulation of FUC_A could have biological consequences (Mackiewicz & Mackiewicz, 1995). In this present study, there was a significantly higher level of FUC_A in T2DM compared to controls. This result agrees with the findings of other studies. For example, α-1, 3 fucosylation in bi, tri and tetra-antennary N-glycans was increased in acute and chronic inflammation (Mackiewicz & Mackiewicz, 1995). In addition, α-1, 3 fucosylation of α1-acid glycoprotein (AGP) was associated with type I diabetes mellitus. Further, Mackiewicz and Mackiewicz, (1995) showed that FUC_A was increased in inflammation.

Branching

This present study showed that T2DM individuals had significantly decreased levels of low-branching and increased levels of high branching plasma N-glycans **(Table 6.2b)**. The increased branching could be attributed to the increased activity of *N*-acetylglucosaminyltransferase V (GnT V) and the relative abundance of sugar nucleotide donors or co-factors. N-glycosylation is regulated by a nutrient dependent hexosamine biosynthetic pathway (HBP). This pathway yields uridine diphosphate-*N*-acetylglucosamine (UDP-*N*-GlcNAc), a metabolite for N-glycosylation. HBP is part of the metabolic cascade that processes between 3-5% of glucose and its activity is largely dependent on the presence of glucose. As such, a decrease in cellular uptake of glucose

leads to a reduction of UDP-*N*-GlcNAc. Compelling evidence suggests that hyperglycaemia up regulates UDP-N-GlcNAc levels by inducing metabolic flux in the HBP pathway (Keser et al., 2017). UDP-N-GlcNAc in turn, is utilised by different GnTs particularly MGAT4 and MGAT5 to generate high branching N-glycans such as tri-and tetraantennary N-glycans (Buse, 2006; Marshall, Bacote, & Traxinger, 1991; Schleicher & Weigert, 2000; Wellen & Thompson, 2012). Moreover, branching generates extra sites for terminal *N*-acetylneuraminic acid (sialic acid) in a process termed sialylation (Dube & Bertozzi, 2005).

Sialylation

Sialylated glycans are present in cell membranes but their overexpression may indicate vascular tissue damage (Nayak et al., 2008). As is obvious in **Table 6.2b**, there were statistically significantly increased levels of di (S2) and trisialylated (S3) plasma N-glycans among T2DM compared to healthy controls. This is consistent with other studies, which showed that elevated levels of sialic acids were associated with hyperglycaemia (Englyst et al., 2006; Ibrahim et al., 2016; Khalili et al., 2014; Malik, Bashir, Khan, & Iqbal, 2009). Possible explanations for this are that insulin resistance and hyperglycaemia lead to increased production of acute phase proteins and sialyltransferases in the liver and kidneys (Gokmen, Kilicli, Ozcelik, Ture, & Gulen, 2002). Consequently, higher levels of sialic acids are released into the blood stream. In addition, increased levels of sialic acids could be the outcome of increased gluconeogenesis, which in turn leads to elevated phosphoenolpyruvate, a precursor for sialic acid biosynthesis (Ibrahim et al., 2016).

Galactosylation

It has been suggested that galactosylation has an important function in cellular regulation and has several physiological outcomes. For example, agalactosylation of IgG was weakly associated with BMI in Caucasians (Knežević et al., 2010; Perkovic et al., 2014). Similarly, the present study found that loss of galactosylation was correlated with BMI among controls but non-significant after correction for multiple testing **(Supplementary Table S5, See Appendix)** (Perkovic et al., 2014). However, there was no difference in the level of G0 between cases and controls and this may be because BMI was not statistically different in the two groups. Again, studies have shown that loss of galactosylation was associated with ageing (Russell et al., 2017; Lemmers et al., 2017). Likewise, in this present study, G0 was associated with age in both cases and controls. These findings could be due to the fact that ageing results in the decline of β-1,4-

galactosyl transferase (β-1, 4-GalT) or upregulation of β-galactosidase; enzymes that regulate galactose metabolism (Vanhooren et al., 2007; Vanhooren, Laroy, Libert, & Chen, 2008). In addition, compared to controls, T2DM patients had increased G3 N-glycans and reduced A2G. This agrees with the findings by Keser et al., (2017) and shows that higher galactosylation is a characteristic of hyperglycaemic status (Keser et al., 2017).

As stated earlier, differences in glycosylation patterns between T2DM and controls could propel biomarker discovery. Nine GPs (GP1, GP4, GP7, GP11, GP17, GP19, GP22,26, GP29 were found to predict case status using a step-wise Akaike's information criterion (AIC) and Bayesian information criterion (BIC) logistic regression model selection (Table 6.7). This model yielded an area under curve of 80.5% [95% CI 76.4%-84.6%] indicating the predicting power of N-glycans as robust biomarkers **(Tables 6.7, Figures 6.3a & 6.3b)**.

Some of this study's limitations are worth mentioning. Firstly, the sample size was not large and hence the interpretation of the results may not be absolute. Secondly, the reported findings were based on a cross-sectional design and do not represent N-glycan changes over time. Thirdly, a treatment with different medications could have influenced the results among the cases. For example, research showed that oral contraceptives and oestrogen treatment triggered a decrease in branching (Brinkman-Van der Linden et al., 1996) and an increase in core fucosylation among women (Saldova et al., 2012). In addition, non-steroidal anti-inflammatory drug use resulted in a decrease in core-fucosylation and sialyl Lewis x (sLe^x) (Saldova et al., 2012). However, the present study could not control for medication use since T2DM participants were given multiple drugs with different dosages (Adua et al., 2017).

6.7 Conclusion

The application of high throughput HILIC-UPLC has enabled the identification of complex plasma N-glycan structures that are characteristic of T2DM within a West African population, Ghana. Strikingly, many of our findings were similar to Caucasian populations affirming the potentiality of N-glycan profiles as generic and universal biomarkers. Further investigation of N-glycosylation processes in large and distinct populations will be crucial for better understanding of T2DM pathogenesis worldwide and facilitate the development of N-glycan based therapies.

6.8 References

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CHAPTER SEVEN

Prelude

Apart from perceived suboptimal health documented in **Study I (Chapter Four)**, it was also revealed that there was poor control of modifiable risk factors among T2DM sufferers. This finding was surprising especially because all T2DM patients were recruited from the hospital and were known to be on some form of medication. This observation therefore provided enough stimulus for a longitudinal investigation. Accordingly, **Study IV** explored major risk factors among T2DM sufferers, taking into consideration medication usage among T2DM patients over six months. This study has been published in the *Journal of Clinical and Translational Medicine* and it can be cited as:

Adua, E., Roberts, P., Sakyi, S. A., Yeboah, F. A., Dompreh, A., Frimpong, K., ... & Wang, W. (2017). Profiling of cardio-metabolic risk factors and medication utilisation among Type II diabetes patients in Ghana: a prospective cohort study. *Clinical and Translational Medicine*, 6(1), 1-11.

Profiling of cardio-metabolic risk factors and medication utilisation among Type II diabetes patients in Ghana: A prospective cohort study

7.1 Abstract

Type II diabetes mellitus (T2DM) is complicated by multiple cardio-metabolic risk factors. Controlling these factors requires lifestyle modifications alongside utilisation of anti-diabetic medications. Different glucose lowering [(biguanides (BIG), sulfonylureas (SUA), thiazolidinedione (TNZ)], lipid lowering (statins), and anti-hypertensive medicines [angiotensin converting enzyme inhibitors (ACEIs), calcium channel blockers (CCB), angiotensin II receptor blockers (ARB) and central acting drugs (CADs)] have been approved for controlling hyperglycaemia, dyslipidaemia and hypertension respectively. Here, we examined factors that characterise T2DM and explored the response to medication therapy among T2DM patients. This prospective cohort study recruited 241 T2DM patients reporting at a clinic in Ghana, from January to August 2016. Each patient's demographics, medications and anthropometric data was obtained while information on medication adherence was captured using the Morisky adherence scale-8 (MMAS-8). Fasting blood samples were collected for biochemical analysis. The mean age of participants was 57.82 years for baseline and 6-month follow-up. Physical activity differed at baseline and follow up ($p<0.05$) but not body mass index (BMI). BIG alone, or in combination with SUA and TNZ did not improve glycaemic status at follow up ($p>0.05$). Many participants using either ACEI or ARB were able to control their blood pressures. Among dyslipidaemia patients under statin treatment, there was an improved lipid profile at follow-up. Statin medications are effective in reducing dyslipidaemia in T2DM patients. However, control of modifiable risk factors, particularly blood glucose and to a lesser degree blood pressure is suboptimal. Addressing these will require concomitant interventions including education on medication adherence and correct dietary plans, lifestyle modifications and physical activity.

Key words: type II diabetes mellitus, hypertension, anti-diabetic medications, risk factors, Ghana.

7.2 Introduction

Despite substantial efforts, type II diabetes mellitus (T2DM) remains a major contributor to the world's morbidity and mortality (Action to Control Cardiovascular Risk in Diabetes Study Group, 2008; Stratton et al., 2000; Tuomilehto et al., 2001). In 2014 alone, more than 2.2 million people died from the disease and at approximately the same time, nearly 415 million adults were affected worldwide, representing a prevalence rate of ≈ 8.5% (International Diabetes Federation (IDF), 2014; World Health Organisation (WHO), 2014). This prevalence rate is expected to translate into 439 million T2DM cases by 2030 (Epstein, Shepherd, & Kahn, 1999; WHO, 2014; 2015a). Unfortunately, countries with less healthcare resources such as those in sub-Saharan Africa (SSA) are among the most affected with some 14.2 million people presently suffering from the disease (WHO, 2014). For example, in Ghana, T2DM affected more than 266,200 individuals at a prevalence rate of 6% in 2015, and it is presently ranked among the top 10 causes of all adult deaths (Danquah et al., 2012; International Diabetes Federation, 2015).

People with T2DM have an increased risk of developing many health problems such as cardiovascular diseases (Herder, Karakas, & Koenig, 2011; Resnick & Howard, 2002), amputations (Lavery, Armstrong, Wunderlich, Tredwell, & Boulton, 2003; Resnick & Howard, 2002), depression (Campayo, Gómez-Biel, & Lobo, 2011), and cognitive impairment (Action to Control Cardiovascular Risk in Diabetes Study Group, 2008; Biessels, Koffeman, & Scheltens, 2006; Luchsinger, 2012; Zilliox, Chadrasekaran, Kwan, & Russell, 2016). Moreover, prolonged hyperglycaemia is strongly linked with many microvascular and, to a lesser extent, macrovascular complications and premature mortality (DeFronzo & Abdul-Ghani, 2011). In fact, just a 1% rise in glucose level will lead to an 18% increased risk for cardiovascular events (Gerstein et al., 2005), 37% increased risk for renal diseases (Action to Control Cardiovascular Risk in Diabetes Study Group, 2008) and 12-14% increased risk of premature mortality (Gerstein et al., 2005; Stratton et al., 2000).

Additionally, the majority of T2DM patients are physically inactive which has led to dyslipidaemia, obesity and hypertension (Cooper-DeHoff et al., 2010; Echouffo-Tcheugui et al., 2015). These in turn lead to further consequences. Studies have shown that obesity accounts for 14% of all adult deaths while hypertension alone is an independent risk factor for cognitive decline (Reitz et al., 2007), renal dysfunction

(Agarwal & Andersen, 2005; Vaes et al., 2015) and ultimately responsible for 45% of all deaths. Therefore, given these detrimental outcomes, controlling known modifiable factors should be a priority.

It has long been documented that achieving good glycaemic levels is pivotal to delaying T2DM complications. According to the American Diabetes Association (ADA), reduction of microvascular and macrovascular complications is possible at HbA1c < 7% (American Diabetes Association, 2010). This could be achieved with single, combination or multiple glucose lowering medications (Wong et al., 2014).

Alongside maintaining normal glycaemic levels, therapeutic intervention should be extended to target other concomitant factors such as dyslipidaemia, hypertension and obesity (Esposito, Ciotola, Maiorino, & Giugliano, 2008; Nathan et al., 2009). Different lipid lowering and anti-hypertensive drugs have been approved for controlling dyslipidaemia and hypertension respectively; the majority of which are currently available in Ghana (Sarfo et al., 2017). Yet, the control of T2DM modifiable risk factors has been suboptimal, partly because studies to create awareness of T2DM are generally scarce in SSA countries such as Ghana. Moreover, these studies have mainly been cross-sectional, providing limited information on association or causality. Therefore, in this study, we explored the manifestations and the associated factors that characterise T2DM in a longitudinal design. Additionally, this study highlights the proportion of T2DM patients that have good glycaemic control, blood pressure and lipid levels and addresses the factors that contribute to poor blood pressure control, high lipid levels and poor glycaemic status.

7.3 Methods and Study Design

This prospective cohort study was conducted at the diabetic clinic of the Komfo Anokye Teaching Hospital (KATH) from January to August 2016. In all, 241 participants with T2DM aged 35 to 70 years who reported to the clinic for review and medications were recruited. Ethical clearance was approved and written informed consent was obtained from each participant as described in **Chapter Four**.

Inclusion and Exclusion Criteria

The study included only those who were diagnosed as having T2DM, based on the international classification of diabetes (ICD 10) criteria. Participants who were taking insulin injections were assumed to be suffering from type I diabetes mellitus and therefore

were excluded. Additionally, among the original 260 T2DM participants recruited for the study, 19 were excluded, mainly because of missing clinical data.

Anthropometric and Blood Pressure Measurements

After obtaining demographic data and information on the general health status from each participant, information on medication adherence was obtained using the validated Morisky adherence scale-8 (MMAS-8). This questionnaire comprises 8 items and responses for item 1 through 7 are either 'yes' or 'no' whereas item 8 comprises a 5-point Likert scale (Okello et al., 2016). Following this, anthropometric measurements including weight, height, BMI, WHR, SBP and DBP were measured by standard methods as described in **Chapter Four** of this thesis. To assess the level of physical activity, we asked basic questions such "as what is the level of physical activity?" and during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time".

Blood Sample Collection and Biochemical Assay

Venous fasting blood samples were collected from each participant into tubes containing EDTA (ethylene diamine tetraacetic acid), fluoride oxalate and gel separator. Different clinical tests including FPG, HbA1c, TC, HDL-c, LDL-c, TG and VLDL-c were measured on the automated chemistry analyser (Roche Diagnostics, COBAS INTEGRA 400 Plus, USA). Non-HDL and coronary risk (CR) ratios were then calculated. Details are provided in **Chapter Four** of this thesis. Various medications utilised by the T2DM patients at the clinic are shown in **Figure 7.1**.

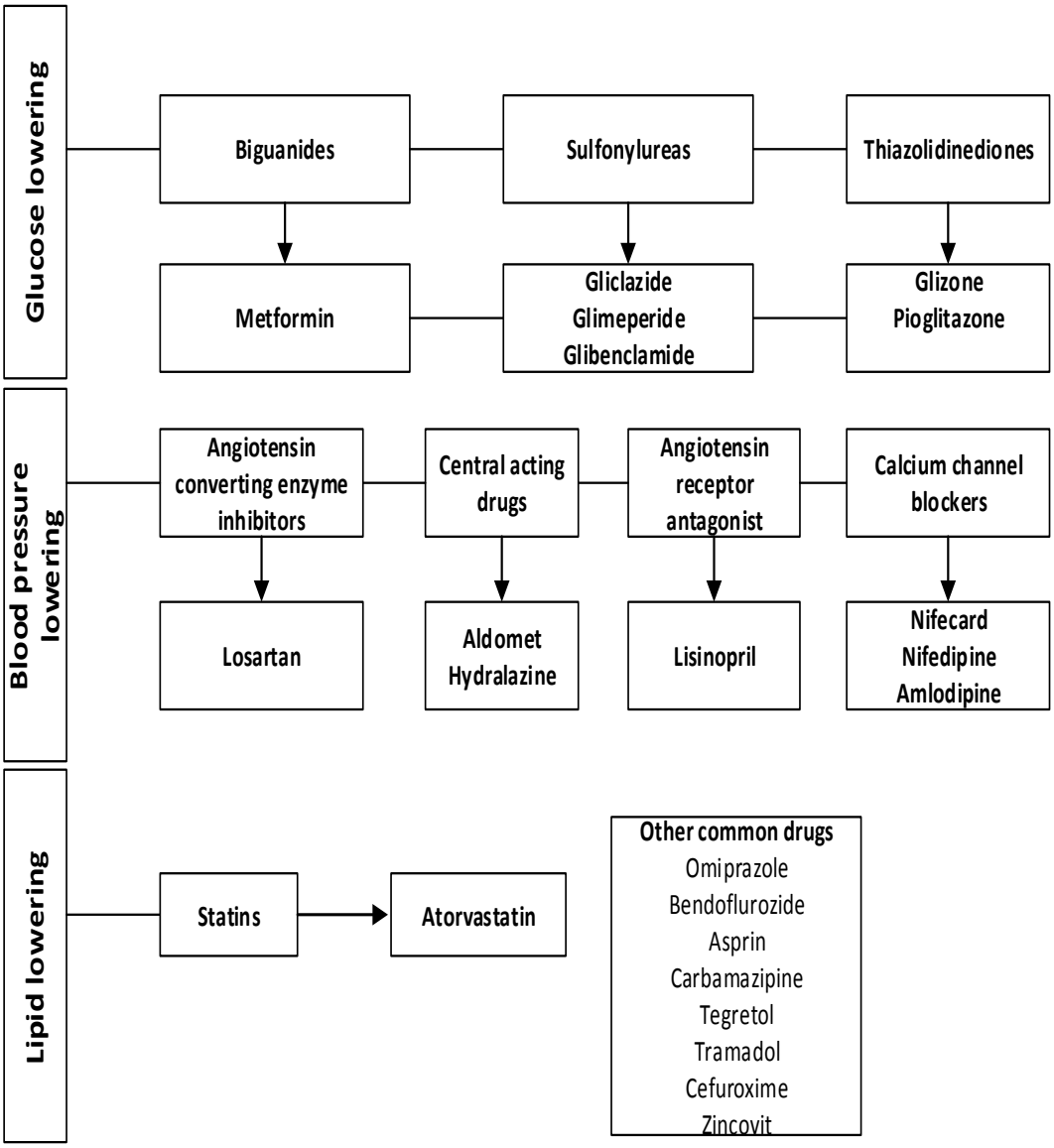

Figure 7.1. Category of medications utilised by T2DM patients. T2DM patients are given different medications including glucose lowering, lipid lowering and blood pressure lowering agents.

Definitions of terms

High plasma glucose; FPG > 7 mmol/L, HbA1c > 7.2 % (WHO, 2015b)

Normal BP <140/90 mmHg, high SBP > 140mmHg, high DBP > 90mmHg (Echouffo-Tcheugui et al., 2015).

Dyslipidaemia: High TG ≥ 1.7 mmol/l, HDL-c < 1.0 (male), 1.03 (female), high LDL-c ≥ 2.59 mmol/l, high total cholesterol ≥ 5.18 mmol/l, high non-HDL ≥ 3.37 mmol/l (Panel, 2002).

7.4 Statistical Analysis

Normality of the distribution was checked using the Kolmogov Smirnov test. All continuous data was recorded as mean ± standard deviation (SD) and as frequency (percentages) for categorical variables. Between groups comparisons for continuous variables were performed using student *t*-tests, and intergroup comparisons of categorical variables were performed using chi-square tests. Association between categorical variables and FPG or HbA1c were performed using logistic regression models and odds ratios (ORs) at 95% confidence intervals (95% CI) were recorded. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS), version 22. A $p<0.05$ was considered significant.

7.5 Results

Among the study population, the male to female ratio was 99/142 at baseline and 66/94 at follow up respectively. BMI and WHR of participants did not significantly differ from baseline to follow up [i.e. ($p=0.172$) and ($p=0.276$) respectively]. However, there was a significant difference in levels of physical activity from baseline to 6-month follow up ($p = 0.0001$) **(Table 7.1)**.

Table 7.1 Socio-demographic characteristics of study participants: Baseline and follow-up

Variable	Total	Baseline (n = 240)	Follow up (n = 160)	X^2, df	p-value
Age (years)	57.80 ± 10.63	57.82 ± 10.88	57.79 ± 10.39	0.370t	0.981
Male: Female ratio	165/236	99/142	66/94		
BMI (Kg/m2)	26.80 ± 9.44	26.13 ± 5.11	27.47 ± 13.78	1.367t	0.172
WHR	0.93 ± 0.05	0.93 ± 0.06	0.92 ± 0.05	1.090t	0.276
Marital status				17.5, 3	0.002
Married	269 (67.1)	164 (68.0)	105 (65.6)		
Never married	6(1.5)	4 (1.7)	2(1.3)		
Divorced	41 (10.2)	25 (10.4)	16 (10.0)		
Widowed	85 (21.2)	48 (19.9)	37 (23.1)		
Education				3.01, 4	0.55
Tertiary	58 (14.5)	36 (14.9)	22 (13.8)		
Senior high school	104(25.9)	57 (23.7)	47 (29.4)		
Junior high school	133(33.2)	78 (32.4)	55 (34.4)		
Lower primary	43(10.7)	28 (11.6)	15(9.4)		
No formal education	63(15.7)	42 (17.4)	21(13.1)		
Occupation				39.65, 3	0.0001
Employed	229(57.1)	133(55.2)	96(60.0)		
Retired	85(21.2)	35(14.5)	50(31.1)		
Unemployed	65(16.2)	51(21.1)	14(8.8)		
Informal employment	22(5.5)	21(9.0)	1(1)		
Physical activity				25.22, 1	0.0001
Primarily sedentary	101(25.2)	79(32.8)	22(13.8)		
Moderate activity	300(74.6)	162(67.2)	138(85.5)		

Values are presented as frequency (percentage); mean ± SD; ^{t}:t-test value

The majority of the study participants were aged 51-60 years [81 (33.6%) vs 55 (34.4%)] while the lowest age range was 31-40 years [14 (5.8%) vs 10 (6.3%)] **(Table 7.2)**. The severity and mean levels of the measured parameters were not significantly different from baseline to follow-up; [SBP (p=0.474 and p=0.600), DBP (p=0.382 and p=0.620), FPG (p=0.364 and p=0.940), TC (p=0.328 and p=0.160), non-HDL-c (p=0.270 and p=0.250) and LDL-c (p=0.092 and p=0.430)]. However, there was a difference in the severity and mean levels of HbA1c [(p=0.004 and p=0.0001)], TG [(p=0.006 and p=0.0001)] and HDL-c [(p<0.0001 and p=0.0001)] from baseline to follow up **(Table 7.2)**.

Table 7.2 Distribution of clinical characteristics among study participants

Variables	Total	Baseline	Follow-up	X^2 , df	P
Age				0.909, 4	1.000
31-40	24(6.0)	14(5.8)	10(6.3)		
41-50	76(19.0)	49(20.3)	27(16.9)		
51-60	136(33.9)	81 (33.6)	55(34.4)		
61-70	118(29.4)	68(28.2)	50(31.3)		
71-80	47(11.7)	29(12.0)	18(11.3)		
BMI				3.386, 3	0.336
Underweight	11(2.8)	9(3.80)	2(1.30)		
Normal weight	175(43.9)	170(44.6)	68(42.8)		
Overweight	132(33.10)	80(33.3)	52(32.7)		
Obese	81(20.3)	44(18.3)	37(23.3)		
SBP				0.028, 1	0.474
Normal	121 (55.1)	132 (54.8)	89 (55.6)		
High	180 (44.9)	109 (45.2)	71 (44.4)		
DBP				0.178, 1	0.382
Normal	298 (74.5)	177 (73.8)	121 (75.6)		
High	102 (25.5)	63(26.3)	39 (24.4)		
HbA1c				7.280, 1	**0.004**
Normal	104(26.0)	74 (30.8)	30 (18.8)		
High	296 (74.0)	166 (69.2)	130 (81.3)		
FPG				0.202, 1	0.364
Normal	160 (39.9)	94(39.0)	66(41.3)		
High	241 (60.1)	147 (61.0)	94(58.8)		
TG				6.679, 1	**0.006**
Good	343(86.2)	199(82.6)	144(91.7)		
High	55(13.8)	42(17.4)	13(8.3)		
TC				0.308, 1	0.328
Good	259 (65.2)	154 (64.2)	105(66.9)		
High	138(34.8)	86(35.8)	52(33.1)		
HDL				94.80, 1	**<0.0001**
Good	235 (59.0)	189(78.4)	46(29.3)		
Low	163(41.0)	52(21.6)	111(70.7)		
NonHDL				0.474, 1	0.270
Normal	188(47.4)	117(48.8)	71(45.2)		
High	209(52.6)	123(51.3)	86(54.8)		
LDL				2.040, 1	0.092
Good	164(41.3)	106(44.2)	58(36.9)		
High	233(58.7)	134(55.8)	99(63.1)		
SBP (mmHg)	140.06 ± 24.09	139.41 ± 24.31	140.71 ± 23.88	0.525 ^{t}	0.600
DBP (mmHg)	81.96 ± 13.18	81.63 ± 13.71	82.28 ± 12.65	0.484 ^{t}	0.620
FPG (mmol/l)	18.32 ± 4.31	9.18 ± 4.42	9.14 ± 4.20	0.082 ^{t}	0.940
HbA1c (mmol/l)	8.79 ± 2.49	8.27 ± 2.10	9.32 ± 2.88	4.201 ^{t}	**0.0001**
TC (mmol/l)	4.63 ± 1.27	4.73 ± 1.27	4.54 ± 1.27	1.406 ^{t}	0.160
TG (mmol/l)	1.17 ± 0.56	1.27 ± 0.57	1.07 ± 0.56	3.520 ^{t}	**0.0001**
HDL-c (mmol/l)	1.19 ± 1.19	1.35 ± 1.35	1.03 ± 1.03	9.960 ^{t}	**0.0001**
Non-HDL-c (mmol/l)	3.44 ± 1.22	3.37 ± 1.24	3.52 ± 1.20	1.142 ^{t}	0.250
LDL-c (mmol/l)	2.91 ± 0.57	2.79 ± 1.16	3.03 ± 1.13	2.029 ^{t}	0.430

Values expressed as Mean ± SD, X^2=Chi-Square test, ^{t} t-statistic, test of statistical significance is two tailed and are bolded.

Table 7.3 demonstrates that after adjusting for age and medication use, high BMI, SBP, DBP, TC, TG, HDL-c, non-HDL-c, and LDL-c status were not significant independent risk factors for high FPG in both baseline and follow-up groups (p>0.05).

Table 7.3 Association between metabolic risk factors and FPG levels at baseline and follow-up

Variables	FPG (Baseline)		X^2, df (p-value)	p	FPG (Follow-up)		X^2, df (p-value)	p
	High (n=147)	Normal (n=94)	aOR (95% CI)		High (n=94)	Normal (n=66)	aOR (95% CI)	
Gender			1.38, 1 (0.239)				3.55, 1(0.06)	
Male	56(38.1)	43(45.7)	1.0#		33(35.1)	33(50.0)	1.0#	
Female	91(61.9)	51(54.3)	1.37(0.81-2.32)	0.283	61(64.9)	33(50.0)	1.85(0.97-3.51)	0.073
BMI			2.18, 3 (0.537)				1.77, 3(0.622)	
Underweight	6(4.1)	3(3.2)	1.05(0.25-4.47)	1.000	2(2.1)	1(1.5)	1.40(0.12-16.21)	1.000
Normal	70(47.9)	37(39.4)	1.0#		40(42.6)	28(43.1)	1.0#	
Overweight	46(31.5)	34(36.2)	0.72(0.39-1.29)	0.289	29(30.9)	23(35.4)	0.88 (0.43-1.83)	0.852
Obese	24(16.4)	20(21.3)	0.63(0.31-1.30)	0.268	23(24.5)	14(21.5)	1.15(0.51-2.62)	0.836
SBP			0.87, 1 (0.351)				1.13, 1(0.288)	
Normal	77(52.4)	55(58.5)	1.0#		49(52.1)	40(60.6)	1.0#	
High	70(47.6)	39(41.5)	1.28(0.76-2.16)	0.357	45(47.9)	26(39.4)	1.41(0.75-2.68)	0.333
DBP			0.02, 1(0.901)				2.34, 1(0.126)	
Normal	108(73.5)	69(74.2)	1.0#		67(73.1)	54(81.8)	1.0#	
High	39(26.5)	24(25.8)	1.04(0.57-1.88)	1.000	27(28.7)	12(18.2)	1.81(0.84-3.91)	0.139
TC			0.22, 1(0.642)				0.09, 1 (0.764)	
Good	92(63.0)	62(66.0)	1.0#		62(66.0)	43(68.3)	1.0#	
High	54(37.0)	32(34.0)	1.14(0.67-1.96)	0.681	32(34.0)	20(31.7)	1.11(0.56-2.19)	0.863
TG			0.23, 1(0.630)				0.52,1(0.472)	
Good	120(81.6)	79(84.0)	1.0#		85(90.4)	59(93.7)	1.0#	
High	27(18.4)	15(16.0)	1.19(0.59-2.37)	0.729	9(9.6)	4(6.3)	1.56(0.46-5.31)	0.565
HDL-c			0.01, 1(0.928)				0.83,1(0.363)	
Good	115(78.2)	74(78.7)	1.0#		25(26.6)	21(33.3)	1.0#	
Low	32(21.8)	20(21.30)	1.03(0.55-1.93)	1.000	69(73.4)	42(66.7)	1.38(0.69-2.77)	0.377
Non-HDL			0.05, 1 (0.827)				0.24,1(0.621)	
Normal	72(49.3)	45(47.5)	1.0#		41(43.6)	30(47.6)	1.0#	
High	74(50.7)	49(52.1)	0.94(0.56-1.59)	0.895	53(56.4)	33(52.4)	1.18(0.62-2.23)	0.628
LDL-c			0.88, 1 (0.349)				0.84,1 (0.358)	
Good	68(46.6)	38(40.4)	1.0#		32(34.0)	26(41.3)	1.0#	
High	78(53.4)	56(59.6)	0.78(0.46-1.32)	0.355	62(66.0)	37(58.7)	1.36(0.71-2.63)	0.401

X^2, df: chi-square value, degrees of freedom; aOR: adjusted odds ratio; CI: confidence interval. Logistic regression model, adjusted for age and medication.
1.0#: reference point for odds ratio

Table 7.4 demonstrates that after adjusting for age and medication use, high BMI, SBP, DBP, TC, TG, HDL-c, non-HDL-c, and LDL-c status were not significant independent risk factors for high HbA1c in both baseline and follow-up groups (p>0.05).

Table 7.4 Association between metabolic risk factors and HbA1c levels at baseline and follow-up

Variables	**HbA1c (baseline)**		X^2, df (p-value)	p	**HbA1c (follow-up)**		X^2, df (p-value)	p
	Poor (n=167)	Good (n=74)	aOR (95% CI)		Poor (n=130)	Good (n=30)	aOR (95% CI)	
Gender			0.18,1 (0.675)				0.96, 1(0.328)	
Male	67(40.4)	32(43.2)	1.0#		56(43.1)	10(33.3)	1.0#	
Female	99(59.6)	42(56.8)	1.13(0.65-1.96)	0.673	74(56.9)	20(66.7)	0.66(0.29-1.52)	0.413
BMI			1.35,3(0.718)				4.38,3 (0.224)	
Underweight	5(3.0)	4(5.4)	0.49(0.12-1.94)	0.445	2(1.6)	0(0.0)		
Normal	77(46.7)	30(40.5)	1.0#		56(43.4)	12(40.0)	1.0#	
Overweight	53(32.1)	26(35.1)	0.79(0.42-1.49)	0.519	45(34.90	7(23.3)	1.38 (0.50-3.78)	0.619
Obese	30(18.2)	14(18.9)	0.83(0.39-1.79)	0.695	26(20.2)	11(36.7)	0.50(0.19-1.29)	0.216
SBP			2.22,1 (0.136)				0.47, 1(0.491)	
Normal	86(51.8)	46(62.2)	1.0#		74(56.9)	15(50.0)	1.0#	
High SBP	80(48.2)	28(37.8)	1.53(0.87-2.68)	0.161	56(43.1)	15(50.0)	0.76(0.34-1.68)	0.544
DBP			0.16,1(0.692)				0.022, 1 (0.883)	
Normal	121(72.9)	55(75.3)	1.0#		98(75.4)	23(76.7)	1.0#	
High DBP	45(27.1)	18(24.7)	1.14(0.60-2.14)	0.752	32(24.6)	7(23.3)	1.07(0.42-2.73)	1.000
TC			2.42, 1(0.12)				0.49, 1(0.483)	
Good	101(61.2)	53(71.6)	1.0#		84(65.6)	21(72.4)	1.0#	
High	64(38.8)	21(28.4)	1.60(0.88-2.89)	0.144	44(34.4)	8(27.6)	1.38(0.56-3.36)	0.522
TG			0.00,1(0.985)				1.42,1(0.233)	
Good	137(82.5)	61(82.4)	1.0#		119(93.0)	25(86.2)	1.0#	
High	29(17.5)	13(17.6)	0.99(0.48-2.04)	1.000	9(7.0)	4(13.8)	0.47(0.14-1.68)	0.262
HDL-c			0.11,1(0.743)				0.46,1(0.499)	
Good	132 (79.0)	57(77.0)	1.0#		39(30.5)	7(24.1)	1.0#	
Low	35(21.0)	17(23.0)	0.90(0.46-1.73)	0.737	89(69.5)	22(75.9)	0.73(0.29-1.84)	0.652
Non-HDL			0.25,1(0.620)				0.002,1 (0.962)	
Normal	79(47.9)	38(51.4)	1.0#		58(45.3)	13(44.8)	1.0#	
High	86(52.1)	36(48.6)	1.15(0.66-1.99)	0.675	70(54.7)	16(55.2)	0.98(0.44-2.21)	1.000
LDL-c			0.05,1(0.817)				0.53,1(0.465)	
Good	74(44.8)	32(43.2)	1.0#		49(38.3)	9(31.0)	1.0#	
High	91(55.2)	42(56.8)	0.94(0.54-1.630	0.888	79(61.7)	20(69.0)	0.73(0.31-1.72)	0.528

X^2, df: chi-square value, degrees of freedom; aOR: adjusted odds ratio; CI: confidence interval. Logistic regression model, adjusted for age and medication. 1.0#: reference point for odds ratio.

From baseline to follow-up, FPG levels increased by 25.0% when (BIG) was administered alone. In a combination therapy with either SUA or TNZ, there was only a decrease in FPG levels by 1% (p=0.9924) and 1.6% (p=0.1098) respectively. However, FPG levels decreased by 15.8% when all three medications; BIG, SUA and TNZ were administered (p=0.216). Meanwhile, levels of HbA1c were increased by 29.6% after BIG treatment alone (p=0.0094), increased by 19.2% and 16.7% when BIG was combined with SUA (p=0.0175) and TNZ (p=0.0903) respectively. However, a multiple therapy of BIG, SUA and TNZ resulted in only a 1.3% increase in HbA1c levels (p=0.8308) **(Table 7.5)**.

Table 7.5 Utilisation of glucose lowering medications among T2DM patients

Treatment	Baseline	Follow up	Mean difference (95% CI)	P	% effect
	FPG (mmol/l)				
BIG only	8.02 ± 0.65	10.08 ± 1.12	2.05(-1.25 to 5.36)	0.2162	25.00%
BIG+ SUA	8.45 ± 0.49	8.441 ± 0.82	-0.01(-1.83 to 1.82)	0.9924	-0.10%
BIG+TNZ	9.63 ± 0.59	11.88 ± 1.47	2.25 (-0.52 to 5.02)	0.1098	23.40%
BIG+ SUA+TNZ	9.921 ± 0.66	8.36 ± 1.04	-1.57 (-4.06 to 0.93)	0.216	-15.80%
	HbA1c (%)				
BIG only	7.34 ± 0.28	9.51 ± 1.10	2.17 (0.57 to 3.78)	**0.0094**	29.60%
BIG+ SUA	8.11 ± 0.32	9.67 ± 0.65	1.55 (0.28 to 2.83)	**0.0175**	19.20%
BIG+TNZ	8.68 ± 0.33	10.12 ± 1.04	1.45 (-0.23 to 3.14)	0.0903	16.70%
BIG+SUA+TNZ	8.46 ± 0.26	8.57 ± 0.47	0.11 (-0.91 to 1.12)	0.8308	1.30%

BIG-Biguanide; SUA-Sulfonylurea; TNZ- Thiazolidinedione. p<0.05 is considered significant and are bolded.

There was a mean percentage decrease effect in levels of HDL-c (p<0.0001), TG (p=0.0259) and VLDL-c (p=0.0237) by 22.8%, 18.4% and 17.3% respectively, after atorvastatin treatment alone. Conversely, there was an increased effect in levels of TC (p=0.743) by 1.7%, non-HDL-c (p=0.075) by 14.5%, LDL-c (p=0.022) by 21.5% and CR (p=0.955) by 0.5% after atorvastatin treatment **(Table 7.6)**.

Table 7.6 Utilisation of lipid lowering medications among T2DM patients

Variable	Baseline	Follow up	Mean difference (95% CI)	P	% difference
TC (mmol/l)					
No statin	5.03 ± 0.12	4.95 ± 0.13	-0.07 (-0.42 to 0.28)	0.6817	1.39%
Atorvastatin	4.06 ± 0.16	4.13 ± 0.16	0.07 (-0.37 to 0.51)	0.7434	1.72%
TG (mmol/l)					
No statin	1.32 ± 0.06	1.19 ± 0.06	-0.13(-0.30 to 0.04)	0.1322	9.85%
Atorvastatin	1.14 ± 0.06	0.93 ± 0.06	-0.21(-0.39 to -0.03)	**0.0259**	18.42%
HDL-c (mmol/l)					
No statin	1.36 ± 0.03	1.05 ± 0.03	-0.30(-0.39 to -0.21)	< **0.0001**	22.06%
Atorvastatin	1.36 ± 0.04	1.04 ± 0.04	-0.31(-0.42 to -0.19)	< **0.0001**	22.79%
Non-HDL (mmol/l)					
No statin	3.67 ± 0.11	3.89 ± 0.12	0.23(-0.09 to 0.55)	0.1617	6.27%
Atorvastatin	2.69 ± 0.15	3.09 ± 0.15	0.39(-0.04 to 0.81)	0.0754	14.50%
LDL-c (mmol/l)					
No statin	3.06 ± 0.11	3.36 ± 0.11	0.30(-0.01 to 0.60)	0.058	9.80%
Atorvastatin	2.19 ± 0.15	2.68 ± 0.14	0.47(0.06 to 0.87)	**0.022**	21.46%
CR					
No statin	5.24 ± 0.15	5.57 ± 0.49	0.33(-0.68 to 1.34)	0.5202	6.29%
Atorvastatin	4.31 ± 0.19	4.32 ± 0.20	0.02(-0.53 to 0.57)	0.9547	0.46%
VLDL-c (mmol/l)					
No statin	0.60 ± 0.03	0.58 ± 0.05	-0.01(-0.12 to 0.09)	0.8181	1.67%
Atorvastatin	0.52 ± 0.03	0.42 ± 0.03	-0.09(-0.18 to -0.01)	**0.0237**	17.3%

CI: confidence interval, $p<0.05$ is considered significant and are bolded.

For non-hypertensive T2DM participants, there was neither a significant change in SBP nor DBP from baseline to follow-up **(Table 7.7)**. SBP levels were reduced by 0.1% after CCB+ACEI treatment (p=0.969). Levels of both SBP and DBP were reduced by 1.9% (p=0.644) and 5.8% (p=0.128) respectively after ACEI treatment alone and decreased by 1.0% (p=0.835) and 0.1% (p=0.912) respectively after CCB + ARB combination therapies. However, levels of both SBP and DBP increased by 3.0% (p=0.683) and 0.4% (p=0.942) respectively after CCB treatment alone and increased by 17.3% (p=0.061) and 11.3% (p=0.086) respectively after CAD treatment alone, while a combination therapy of CCB+ACEI increased DBP by 1.9% (p=0.666) **(Table 7.7)**.

Table 7.7 Utilisation of anti-hypertensive medicines among T2DM patients.

Anti-hypertensive drugs	Baseline	Follow up	Mean difference (95%CI)	p-value	%effect
	SBP (mmHg)				
DM only (n=38)	127.1 ± 4.09	130.5 ± 3.45	3.34(-7.33 to 14.01)	0.534	2.63%
DM+HPT	150.2 ± 7.99	154.6 ± 7.21		0.683	2.96%
CCB (n=11)			4.46(-18.02 to 26.92)		
ARB (n=22)	130.1 ± 3.04	130.0 ± 4.36	0.01(-10.74 to 10.74)	> 0.999	0.00%
ACEI (n=30)	130.4 ± 3.93	128.0 ± 3.59	-2.47(-13.12 to 8.19)	0.644	-1.90%
CAD (n=8)	150.6 ± 9.07	176.6 ± 8.33	26.0(-2.43 to 54.43)	0.061	17.30%
CCB+ ARB (n=24)	153.3 ± 5.74	151.7 ± 4.98	-1.58(-16.88 to 13.72)	0.835	1.03%
CCB+ACEI (n=27)	143.1 ± 3.33	142.9 ± 3.46	-0.19(-9.83 to 9.46)	0.969	0.13%
	DBP (mmHg)				
DM only (n=38)	74.87 ± 2.25	77.87 ± 1.80	3.00(-2.75 to 8.74)	0.301	4.00%
DM+HPT CCB (n=11)	83.18 ± 3.74	83.55 ± 3.23	0.36(-9.95 to 10.68)	0.942	0.43%
ARB (n=22)	80.00 ± 2.31	80.02 ± 2.09	1.00(-5.29 to 7.30)	0.75	1.25%
ACEI (n=30)	80.01 ± 1.89	76.43 ± 2.37	-4.67(-10.73 to 1.40)	0.128	5.76%
CAD (n=8)	93.80 ± 5.23	104.4 ± 7.22	10.6(-9.96 to 31.16)	0.086	11.30%
CCB+ ARB (n=24)	86.13 ± 3.33	86.08 ± 2.62	-0.04(-8.58 to 8.50)	0.992	0.05%
CCB+ACEI (n=27)	82.81 ± 2.78	84.41 ± 2.40	1.59(-5.78 to 8.96)	0.666	1.92%

CCB-calcium channel blockers; ACEI-angiotensin converting enzyme inhibitors; ARB-angiotensin II receptor blockers; CAD-central acting drugs.

7.7 Discussion

The prevalence of T2DM has increased tremendously in the past few decades among different countries worldwide (Adua, Frimpong, Li, & Wang, 2017; Asweto, Alzain, Andrea, Alexander, & Wang, 2016; WHO, 2014; Wang, 2016). SSA remains one of the most affected regions due to rapid urbanisation and increased adoption of a westernised diet with less physical activity (Danquah et al., 2012; Frank et al., 2014).

In this present hospital-based study, we examined the major risk factors that characterise T2DM and how these factors influence anti-diabetes medication response. As reported by Danquah et al., (Danquah et al., 2012), the majority of T2DM patients in urban Ghana are middle aged, of low socio-economic status and their lifestyle is primarily sedentary (Danquah et al., 2012). Moreover, our findings on clinical parameters such as SBP, DBP, HDL-c, LDL-c, TG, TC and FPG are similar to those reported in their study (Danquah et al., 2012).

Overall, several of these biomarkers are higher than the recommended threshold for T2DM as suggested by the WHO and the ADA (American Diabetes Association, 2015; WHO, 2006). For example, approximately 60% and 69.2% of the participants

were not able to achieve the desired FPG and HbA1c targets respectively. This is in fact disturbing given the direct association between abnormal plasma glucose levels and macrovascular and or microvascular complications. Efforts to control glucose levels are necessary and could be achieved in several ways. After diagnosis, medical nutrition therapy (MNT) is necessary to reduce weight and normalise glucose levels (Esposito et al., 2008; Esposito et al., 2010). However, it has been established that MNT alone is not sufficient for improving plasma glucose levels. As such, the use of medications becomes the next phase of intervention (Esposito et al., 2008).

In Ghana, several glucose lowering medications have been approved for the treatment of hyperglycaemia including SUAs, TNZs and biguanides, the latter being the first line anti-diabetic medicine. Like many other countries, its popularity is because: 1) its less expensive, 2) its effective for weight reduction and plasma glucose levels, and 3) it has a reduced risk for hypoglycaemia (Wong et al., 2014). Not surprisingly, a high proportion of our participants (>80%) were on this medication, most of whom had used this drug for a period long before the start of this study. However, the majority of those who used metformin alone could not achieve the desired glycaemic level even at follow up although there seems to be a minimal percentage effect (29.6%, p=0.0094) on HbA1c level **(Tables 7.3-7.5)**. This emphasises the failure of metformin as a monotherapy to achieve glucose control. At this point, the focus shifts towards individuals undergoing combination and multiple therapies. SUAs and TNZs have been recognised as second line anti-diabetic medications and their efficacy is similar to metformin (Cox & Feinglos, 2013; Wong et al., 2014). However, it was apparent after six months that even with multiple therapies; the majority of the patients could not attain the desired glucose target levels. Only a minimal percentage effect of BIG+SUA (19.2%, p=0.0175) on HbA1c was observed **(Table 7.5)**. Several reasons can be attributed to this:

Firstly, there is a possibility of poor adherence to oral medications, especially among those taking combination and multiple therapies, not only for hyperglycaemia but also for other comorbidities (Emslie-Smith, Dowall, & Morris, 2003). Moreover, many of these drugs are associated with side effects and hence it is possible that some participants will be selective in their choice of medication. In a study among 2,849 T2DM patients in the UK, it was shown that only 13% of the patients adhered strictly to the drug regimen (Donnan, MacDonald, & Morris, 2002). This could possibly be the case in our study as some participants may have become bored with swallowing

different medications daily. Efforts to simplify treatment regimens should therefore be intensified. For example, instead of multiple medications, single-dose combination pills with minimal side effects could be administered.

Secondly, ensuring adequate control of glycaemic status requires a paradigm shift from sedentary behaviour to a more physically active lifestyle. One study has shown that moderate-intensity physical activity such as brisk walking and reducing time spent watching television to less than 30 minutes per day could reduce several modifiable T2DM risk factors including plasma LDL-c and TG while increasing HDL-c (Laaksonen et al., 2002). A meta-analysis also showed that physical activity is inversely associated with risk for T2DM (Aune et al., 2015). Moreover, intense exercise is necessary to stimulate 5-adenosine monophosphate-activated kinase (5-AMPK) causing the release of glucose to the muscles rather than it accumulating in the plasma (Epstein et al., 1999). In our study however, we were unable to assess the level or intensity of physical activity by the individuals. Therefore, an effective physical assessment tool such as the international physical activity questionnaire (IPAQ) could be useful (Lee et al., 2011).

Thirdly, poor dietary preferences may have been a contributory factor. Studies have shown that healthy diets or consumption of vegetables, low calorie diets, low trans fats, legumes, fruits, poultry, whole grains and cereal fibre is linked to a reduced risk of metabolic syndrome and T2DM (Frank et al., 2014). Conversely, consumption of red and processed meats, sweets, desserts and fried foods is associated with an increased risk of T2DM (Esposito et al., 2008; Frank et al., 2014). However, whether or not the majority of the study participants utilised a particular food was unverified and therefore, a validated food frequency questionnaire would have been useful.

Despite the increasing use of anti-hypertensives, BP control was suboptimal in our study population. With an attrition rate of nearly 40%, only 52 T2DM participants who took anti-hypertensive medications were able to maintain a target BP (both SBP and DBP) at follow up **(Table 7.7)**. The majority were unable to achieve a desired target although they took more than one antihypertensive drug. This is a striking result given that high BP is by far the most critical risk factor for cardiovascular disease (CVD) and stroke (Zhou, & Cai, 2011). Studies that explore the role of intensive BP control in preventing CVD have produced conflicting results. One study showed that a DBP of $\leq$ 80 mmHg could reduce the risk of CVD by 50% (Hansson et al., 1998). However, another study reported that SBP $\leq$ 120 mmHg was not associated with a

reduced risk for CVD (Cooper-DeHoff et al., 2010). Notwithstanding this, our findings agree with several other studies that BP is poorly controlled among T2DM patients worldwide (Cheung et al., 2009; Saydah, Fradkin, & Cowie, 2004).

Statins are well-known lipid lowering medications and the common one used by participants in this study is atorvastatin. More than half of the participants taking atorvastatin had good lipid profiles and this is consistent with the findings by Wong et al., (Wong et al., 2014). Moreover, our study showed that there was a significant improvement in several lipid markers such as TG, LDL-c, HDL-c and VDL-c at follow up **(Table 7.6)**. Whether atorvastatin interfered with glucose homeostasis is yet to be determined but our study confirms that atorvastatin is a potent drug for treating dyslipidaemia.

The present study does have some limitations. Firstly, because it was an observational longitudinal study, it was limited by confounding factors such as differences in dosage regimen. Dosage regimen refers to the modality of drug administration/doses per unit of time to reach a therapeutic objective. This comprises the time or frequency when the drug should be administered, the time between doses and the amount or unit dose of medicine to be administered at a specific time (Claxton, Cramer, & Pierce, 2001; Patrick, 2013; Ritschel & Kearns, 2009). However, given the number of participants, each with a different medication dosage at a point in time, it was difficult to take into consideration the dosage regimen. At the same time, certain tests, especially FPG, are influenced by biological variation even when fluoride tubes are used. For example, stressful situations in the hours preceding a FPG test could increase FPG levels (Bonora & Tuomilehto, 2011). Thus, we were unable to provide a full explanation of the poor drug response among some participants. Secondly, a clinical randomised control trial would have eliminated potential confounding factors, and shed further light on the effect of the various medications in lowering modifiable risk factors. Thirdly, the sample size of the study was small and therefore cannot be representative of the entire T2DM population. Finally, over 40% of the participants were lost to follow-up and this may have had an effect on our assessments.

7.7 Conclusion

This study showed that the use of statins is effective for improving lipid profiles and can be regarded as a potent medication for treating dyslipidaemia in the Ghanaian population. However, utilisation of oral hypoglycaemic agents whether as a monotherapy, combination or polytherapy was not effective for achieving plasma

glucose targets of < 7%. This is alarming and therefore, alternative approaches including a less sedentary lifestyle while engaging in vigorous exercise may reduce weight and obesity; enforcing healthy eating practices and administration of single/fixed-dose combination tablets or pills with minimal side effects may improve medication adherence.

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Chapter 8 is not available in this version of the thesis

CHAPTER NINE

9.1 General Discussion

Study I: Incorporation of suboptimal health status as a potential risk assessment for type II diabetes mellitus: A case-control study in Ghana

Due to the exponential growth of the economy, improvement in living standards, industrialisation and urbanisation, a significant number of people in Ghana suffer from many chronic diseases such as T2DM. A study has indicated that up to 6% of the adult population have T2DM and the disease is responsible for many hospitalisations, huge costs to the government and increased out-of-pocket costs to the taxpayers (Danquah et al., 2012). However, available data suggest that the development of T2DM takes up to 30 years, leaving room for early intervention. Hence, **Study I** applied the SHSQ-25 to reveal those who could develop T2DM over time. It was shown that age, education, physical activity, DBP, SBP and TG levels were significantly associated with high SHS. This result is consistent with previous studies among Chinese and Caucasians and confirms the robustness of SHSQ-25. However, this study could not validate the association between high SHS and FPG, HbA1c, TC, LDL-c and HDL-c as previously found among Chinese populations. In addition, the median SHS score of 22 found in this study was lower than that of the Chinese cohorts. The possible explanation for these discrepancies are the sample size. The previous investigations were mainly conducted among large Chinese cohorts, i.e. 2,799 participants in 2009 (Yan et al., 2009), 3,019 in 2012 (Yan et al., 2012), 3,405 in 2012 (Wang & Yan, 2012) and 4,313 in 2016 (Wang et al., 2016).

Further in **Study I**, the control of risk factors among the T2DM patients was poor and this could be blamed on delayed intervention, ineffective treatments, untargeted medications, drug resistance, poor infrastructure, genetic, epigenetic and environmental factors (Golubnitschaja, 2010; Golubnitschaja et al., 2016; Golubnitschaja et al., 2014; Lemke & Golubnitschaja, 2014). Overall, the findings of this study provide a stimulus for risk prediction, patient stratification, disease modelling, surveillance, optimal diagnosis and prediction of adverse drug-drug interaction.

While this study indicated the usefulness of SHSQ-25 as a subjective marker for risk stratification for T2DM, it was obvious that the biological underpinnings of T2DM could not be unravelled with the SHSQ-25 alone. It was necessary to complement it with robust objective markers, and this led to **Study II**.

Study II: Utilisation of N-glycosylation profiles as risk stratification biomarkers for suboptimal health status and metabolic syndrome

In this study, HILIC-UPLC was used to profile the plasma N-glycome in healthy individuals, as well as those with SHS and MetS. It was found that GP31(FA3G3S[3,3,6]3), p=0.0437)), GP34 A4G4S[3,3,6]3) p=0.0110)), and GP38 (A4G4S[3,3,3,6]4); p=0.0493)) were increased in high SHS. Intriguingly, these associations were obvious in patients with MetS and strengthens the link between high SHS and MetS. Thus, high SHS could be a unique checkpoint for intervention. As stated previously, individuals with high SHS could be targeted for tailored interventions that may postpone the development of MetS or T2DM. This study also demonstrated that N-glycan complexity was linked with MetS. HB, G3, FUC_A, TRIA were all increased in MetS compared to controls. These associations could be attributed to the over expression of N-acetylglucosaminyltransferase, the enzyme that catalyses β 1, 6 GlcNAc branching and drives the inflammatory processes that characterises MetS (Dube & Bertozzi, 2005).

Recently, N-glycomics has gained considerable interest worldwide; yet, studies that focus on inter-population N-glycan profile comparisons are relatively scarce. Moreover, it is proposed that the N-glycosylation machinery is affected by at least 1% of genes (Lauc, 2016; Lauc et al., 2010). Thus, the pattern of glycosylation across multiple populations may change. Therefore, the findings of this study were compared to those from Chinese Han, Croatian and Orcadian populations. It was revealed that N-glycans not only correlated with biochemical/clinical measures of MetS but also the pattern of N-glycan associations were similar across multiple populations. For example, G3, S3 and TRIA were positively associated with BMI in all four populations (i.e. Ghanaians, Scottish, Chinese and Croatians) **(Table 5.5a)**. This study therefore confirms the potential of N-glycosylation as a generic and universal biomarker for MetS. However, it should be noted that the direction of association was not always the same across the different populations. This is largely because the various populations may be exposed to different environmental factors and stressors. For example, the climatic conditions within the African population are different from those in the Asian or Caucasian populations and this may affect the N-glycosylation process.

While glycomics is a relatively new emerging field, it holds great potential in helping us to understand the pathophysiology of diabetes. However, it is expensive at this stage. The SHS promises a much less expensive means of revealing those at-risk of T2DM but this needs to be validated in very large longitudinal studies. Already,

SHS studies are being conducted among 50,000 Chinese individuals and it is hoped that a clearer understanding of the link between SHS and MetS or T2DM will be revealed (Wang, Russell and Yan, 2014).

Study III: High-throughput profiling of whole plasma N-glycans in Type II diabetes mellitus patients and healthy individuals

The aim of this study was to determine the N-glycan changes that exist in people with T2DM when compared to healthy individuals. It was revealed that N-glycan structures in healthy individuals differed significantly from T2DM patients, and that T2DM was associated with increased plasma N-glycosylation structures. Specifically, and similar to the findings of **Study II**, the present study revealed increased HB, tri-galactosylated (G3), antennary fucosylated (FUC_A) and triantennary (TA) N-glycan structures was associated with T2DM. These findings agree with other studies (Keser et al., 2017; McLachlan et al., 2016), confirming that N-glycan complexity is linked with poor plasma glucose control. There is compelling evidence that reduction of sialylation was associated with chronic diseases including colorectal cancers (Theodoratou et al., 2016), chronic kidney disease (Barrios et al., 2016) and SLE (Vučković et al., 2015), whereas it is increased in hypertension (Wang et al., 2016). The results of this study seems to agree with that of Wang et al., (2016) as increased sialylation was associated with T2DM and confirmed the link between hypertension and T2DM. This could be explained by the fact that during the hyperglycaemic state, there is an increased expression of sialyltransferase (Gokmen et al., 2001). Consequently, more sialic acid is synthesised and released into the plasma. In another study involving a European population, it was shown that decreased IgG sialylation was linked to T2DM (Lemmers et al., 2017). However, since the present study was not restricted to IgG, but the whole human plasma N-glycome, we could not investigate this finding. Further, LB, S0, G1, FUC_C, A2G and BA structures were decreased in T2DM compared to controls.

Overall, **Studies I-III** have given an overview of the health status of the Ghanaian population and demonstrated the efficiency of the HILIC-UPLC technique for glycoprofiling. However, the findings of the study were based on a one-time sampling approach (cross-sectional), lacking cause-effect relationship. Hence, **Studies IV and V** were designed in an attempt to reveal small changes that were associated with T2DM progression by observing the clinical/biochemical data of T2DM patients and examining their N-glycan profiles over time.

Study IV: Profiling of cardio-metabolic risk factors and medication utilisation among Type II diabetes patients in Ghana: A prospective cohort study

Renowned health organisations such as the WHO and the IDF have issued guidelines for risk factor control among T2DM (IDF, 2015; WHO, 2005). However, to date, this effort has yielded mixed results. This study therefore sought to determine the prevalence of risk factors, and trends in medication use among T2DM patients in Ghana and to propose better management approaches.

The results of the study showed that more than 80% of the participants were on anti-diabetic medications, as previously documented by Danquah et al., (2012). Despite the substantial use of these medications, the majority of the T2DM patients could not meet targets for reduced blood pressure and blood glucose levels even at 6-month follow-up. For example, the use of BIG alone could cause only a 29.6% effect on HbA1c levels. When BIG was combined with SUA, a 19.2% effect on HbA1c was observed. In parallel, anti-hypertension medication, use normalised the blood pressure for only 52 out of 122 T2DM patients while half could achieve reduced targets for blood lipid levels after atorvastatin use. There is the need for stringent and better management approaches. For example, dosages should be optimised and patient adherence promoted. This should be complemented with lifestyle changes, such as restriction of dietary carbohydrates, increased exercise and smoking cessation. Further, comprehensive medical and clinical rehabilitation services should be accessible to all T2DM patients (Golubnitschaja et al., 2016; Golubnitschaja, Kinkorova, & Costigliola, 2014; Lemke & Golubnitschaja, 2014).

Study V: N-Glycosylation Profiling of Type II Diabetes Mellitus from Baseline to Follow-up

To date, studies on the stability of plasma N-glycans have only been reported in two studies (Gornik et al., 2009; Hennig et al., 2016). However, their conclusions were based on only 12 and 5 healthy individuals, respectively, and employed analytical tools that allowed the quantification of a limited number of N-glycan peaks. Therefore, the aim of **Study V** was to determine plasma N-glycosylation patterns among T2DM patients, in relation with environmental factors over a 6-month period. It was shown that at baseline, BMI, education, occupation and WHtR as well as levels of HDL-c differed significantly between males and females. In addition, HDL-c levels for all participants differed significantly from baseline to follow-up while N-glycan structures were gender specific, as described in Lu et al., (2011). In particular, levels

of HB, S2, S3, S4, G3, FUC_A and TRIA were statistically significantly higher in females, whereas LB, S0, S1, G1, FUC_C, BA, BAMS and A2G were statistically significantly higher in males compared to females. After calculating the CVs of the N-glycans at baseline and follow-up, it was shown that intra-individual variability of the N-glycans was small, whereas at the population level, the variability was large. However, none of these results could reach statistical significance. These findings confirm the long-term stability of N-glycan structures as previously documented by Gornik et al., (2009) and Hennig et al., (2016). There are discrepancies in the half-life of glycoproteins. In a quantitative study of 39 glycoproteins, it was shown that human cell surface glycoproteins had a median half-life of 19.6 hr (Xiao and Wu, 2017). In another study, it was found that the half-life of more than 20% of glycoproteins exceeded 100hr (Sun et al., 2016). On the other hand, the half-life of IgG in the plasma is almost 26 days with an average plasma concentration of 15g/L days (Novokmet et al., 2014). Given these half-lives, it appears unlikely that there would be significant changes the whole plasma N-glycome through the degradation of old glycans and regeneration of new ones.

Moreover, it is worth noting that N-glycan structures may have changed in the presence of T2DM co-morbidities such as neuropathy, glaucoma, myocardial infarction, coronary artery disease, retinopathy and peripheral ulcers (Testa et al., 2015). However, patients who had these complications did not meet the inclusion criteria of this study. Thus, the lack of change of N-glycan structures was expected. This study highlights the inclusion of N-glycans for T2DM prognosis as monitoring N-glycan profiles over time could reveal biochemical changes that link T2DM and co-morbidities.

9.2 Limitations and Future Perspectives

Although the present overall project produced a number of publications and manuscripts, it was not devoid of limitations. Firstly, the major limitation relates to the small sample size. Moreover, a large number of participants were lost to follow-up while a significant volume of missing data was seen. Combined, these introduced bias and loss of statistical power. Larger cohorts should be considered in future studies, and to compensate for dropouts, multiple imputation of the baseline data should be made. Secondly, there were higher numbers of females than males in both cases and controls and this may have introduced some bias. Thirdly, the project was largely a

cross-sectional one; a 6-month follow up study was only possible among T2DM patients. As such, the project was unable to determine the direction of causality. To better explain this, longitudinal studies are required. Presently, there are ongoing retro (1994-2016) and prospective (2016-2022) longitudinal analyses of plasma N-glycome occurring as part of the Busselton Healthy Ageing Cohort. Results from that study will explain whether changes of N-glycan structure is a cause or a consequence of T2DM.

While there is a possible role for genes in the glycosylation pattern across different populations, the present study could not adequately verify this. However, buffy coat aliquots for these participants are still stored. These samples will be available for GWAS in the near future and could help us to understand the involvement of genes in glycosylation. Increasing evidence suggests that perturbed lipid levels are associated with insulin resistance and T2DM (Boden et al., 2002). This conclusion has largely been accepted in the context of traditional clinical markers including HDL-c, LDL-c, TC, and TG as well as body fat measures such as BMI, WHR and central obesity (Boden et al., 2002). However, the complexity of T2DM cannot be fully described by these markers alone. Hence, a deep profiling of distinct lipid classes, taking into consideration the structure, function and role (lipidomics) is required to provide further insights.

It is known that there is a negative association between hyperglycaemia and the levels of circulating glycocalyx. It is also worth noting the potential impact of high plasma glucose on the glycocalyx volume and increase in vascular vulnerability. As well, other glycocalyx degrading factors including matrix metalloproteinases, sialidases and heparanase may compromise the integrity of the endothelial cells (Sieve, Munster-Kuhnel and Hilfiker, 2018). However, it was beyond the scope of this thesis to measure the levels/volume of glycocalyx or perform such investigations.

Further, the majority of the results from this thesis are associative in nature and mechanistic studies are necessary in the future to understand how N-glycosylation may differ in the population from Ghana.

9.3 General Conclusions

The conclusions from this thesis are: 1) the management of T2DM in Ghana is suboptimal and undiagnosed risk factors remain prevalent, 2) Statin medications are effective for reducing dyslipidaemia in T2DM patients. However, control of modifiable risk factors, particularly blood glucose and to a lesser degree blood pressure is suboptimal, 3) SHS is a significant, albeit modest, risk factor for metabolic

syndrome (MetS) while MetS and T2DM are significantly associated with complex N-glycans. 4) N-glycan structures in T2DM patients are stable over 6 months and may change in the presence of co-morbidities.

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CHAPTER TEN

Appendix I

Sample Size

An estimated sample size of 515 yielded a statistical power of 80%, with an effect size of 0.5 at an alpha level of 0.01 based on power analyses using G*Power software version 3.1.9.2.

Risk Assessment

There was a possibility of cross contamination in the biochemistry laboratory of KATH since different samples were analysed concurrently. However, this was controlled by ensuring quality control measures such as maintaining strict hygienic conditions, proper handling and adequate labelling of samples. Collected blood samples were then frozen at -80^{o}C before transporting to Australia and then to Genos, Croatia for N-glycan analysis. I was aware of possible contamination during sample transportation and so we ensured proper packaging and strict adherence to shipping legislations and requirements.

Figure: A) map of Ghana B) Komfo Anokye Teaching Hospital, Kumasi

Plate Randomisation

Summary (p1) #mean age=54.96, 40.26% F, 1-30 2-29 3-18

Summary (p2) #mean age=55.37, 38.16% F, 1-29 2-28 3-19

Summary (p3) #mean age=53.61, 39.47% F, 1-32 2-28 3-16

Summary (p4) #mean age=55.47, 40.79% F, 1-30 2-26 3-20

Summary (p5) #mean age=54.71, 35.53% F, 1-29 2-31 3-16

Summary (p6) #mean age=56.20, 36.84% F, 1-30 2-29 3-17

Summary (p7) #mean age=55.63, 39.47% F, 1-31 2-29 3-16

Summary (p8) #mean age=54.42, 39.47% F, 1-29 2-28 3-19

Summary (p9) #mean age=54.57, 38.16% F, 1-32 2-28 3-16

R-Packages

1. ggplot2
2. magrittr
3. ggpubr
4. glots
5. rColorBrewer/colorRampPalette
6. heatmap.plus
7. corrgram
8. corrplot
9. devtools
10. easyGgplot2
11. blockrand

Appendix II

Chapter Five Supplementary Data

Supplementary Table 5.1. Derived plasma N-glycan traits calculated from 39 directly measured N-glycan peaks

Variable	Label	Description
LB	Low Branching	GP1+GP2+GP3+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15+GP16+GP17+GP18+GP19+GP20+GP21+GP22+GP23
HB	High Branching	GP24+GP26+GP27+GP28+GP29+GP30+GP31+GP32+GP33+GP34+GP35+GP36+GP37+GP38+GP39
S0	Neutral	GP1+GP2+GP3+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11
S1	Monosialylated	GP12+GP13+GP14+GP15+GP16+GP17
S2	Disialylated	GP18+M9+GP20.21+GP22+GP23+GP24++ GP25+GP26
S3	Trisialylated	GP27+GP28+GP29+GP30+GP31+GP32+GP33+GP34+GP35
S4	Tetrasialylated	GP36+GP37+GP38+GP39
G0	Agalactosylated	GP1+GP2+GP7
G1	Monogalactosylated	GP3+GP4+GP5+GP6+GP12+GP13
G2	Digalactosylated	GP8+GP9+GP10+GP11+GP14+GP15+GP16+GP17+GP18+M9+GP20+GP21+GP22+GP23
G3	Trigalactosylated	GP24+GP26+GP27+GP28+GP29+GP30+GP31+GP32+GP35
G4	Tetragalactosylated	GP33+GP34+GP36+GP37+GP38+GP39
FUC_A	Antennary Fucose	GP32+GP35+GP39
FUC_C	Core Fucose	GP1+GP2+GP4+GP5+GP6+GP10+GP11+GP13+GP16+GP17+GP22+GP23+GP29+GP31
BA	Biantennary	GP1+GP2+GP3+GP4+GP5+GP6+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15+GP16+GP17+GP18+GP20+GP21+GP22+GP23
A2	Biantennary Agalactosylated	GP1+GP2
A2G	Biantennary Galactosylated	GP3+GP4+GP5+GP6+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15+GP16+GP17+GP18+GP20+GP21+GP22+GP23
BAMS	Monosialylated Biantennary	GP12+GP13+GP14+GP15+GP16+GP17
BADS	Disialylated Biantennary	GP18+GP20+GP21+GP22+GP23
TRIA	Triantennary	GP19+GP24+GP26+GP27+GP28+GP29+GP30+GP31+GP32
TA	Tetraantennary	GP33+GP34+GP35+GP36+GP37+GP38+GP39

Supplementary Table 5.2 Distribution of individual plasma N-glycan peaks in low and high SHS

N-glycan	Median(IQR)	Low SHS Range	Median(IQR)	High SHS Range	p-value
GP1	5.92(3.00)	4.52 - 7.52	6.34(2.38)	5.08 - 7.47	0.13049
GP2	2.22(0.73)	1.91 - 2.64	2.34(0.77)	2.04 - 2.81	0.12836
GP3	0.08(0.05)	0.07 - 0.12	0.08(0.04)	0.07 - 0.12	0.58256
GP4	5.19(1.60)	4.34 - 5.94	5.33(1.70)	4.35 - 6.05	0.49885
GP5	2.16(0.69)	1.78 - 2.47	2.14(0.72)	1.76 - 2.48	0.8679
GP6	1.25(0.42)	1.04 - 1.47	1.28(0.36)	1.13 - 1.49	0.19439
GP7	0.98(0.19)	0.90 - 1.1	0.97(0.18)	0.88 - 1.07	0.18638
GP8	1.13(0.27)	1.04 - 1.31	1.14(0.34)	0.99 - 1.34	0.5872
GP9	0.10(0.03)	0.09 - 0.12	0.10(0.02)	0.09 - 0.12	0.92533
GP10	4.62(1.68)	3.67 - 5.35	4.37(1.52)	3.47 - 5	0.07834
GP11	0.81(0.24)	0.69 - 0.94	0.78(0.20)	0.69 - 0.89	0.38403
GP12	0.98(0.16)	0.90 - 1.06	0.97(0.17)	0.89 - 1.06	0.37761
GP13	0.84(0.27)	0.72 - 0.99	0.84(0.26)	0.72 - 0.98	0.87322
GP14	10.48(1.46)	9.76 - 11.23	10.42(1.66)	9.54 - 11.2	0.49885
GP15	0.36(0.08)	0.34 - 0.42	0.37(0.08)	0.34 - 0.43	0.36765
GP16	6.20(1.65)	5.47 - 7.12	6.02(1.42)	5.38 - 6.8	0.14434
GP17	1.77(0.58)	1.43 - 2.01	1.74(0.45)	1.51 - 1.96	0.8892
GP18	3.57(0.77)	3.21 - 3.98	3.44(0.65)	3.18 - 3.84	0.25261
GP19	1.10(0.19)	1.02 - 1.21	1.11(0.23)	1.00 - 1.23	0.94413
GP20	25.24(3.79)	23.68 - 27.47	25.01(3.38)	23.35 - 26.74	0.23485
GP21	0.51(0.13)	0.45 - 0.59	0.50(0.12)	0.45 - 0.58	0.71215
GP22	4.17(0.92)	3.77 - 4.7	4.21(1.00)	3.80 - 4.81	0.4428
GP23	1.94(0.57)	1.66 - 2.24	1.99(0.76)	1.68 - 2.44	0.21864
GP24	1.66(0.58)	1.35 - 1.93	1.64(0.52)	1.36 - 1.89	0.86259
GP25	0.14(0.04)	0.13 - 0.17	0.14(0.05)	0.12 - 0.18	0.8033
GP26	1.46(0.45)	1.23 - 1.68	1.47(0.43)	1.28 - 1.72	0.40275
GP27	0.43(0.29)	0.30 - 0.6	0.46(0.29)	0.33 - 0.62	0.57678
GP28	0.77(0.30)	0.64 - 0.94	0.77(0.23)	0.67 - 0.91	0.92265
GP29	0.19(0.05)	0.18 - 0.23	0.20(0.05)	0.18 - 0.24	0.62973
GP30	5.49(1.82)	4.51 - 6.34	5.67(2.07)	4.46 - 6.54	0.58488
GP31	0.46(0.18)	0.37 - 0.55	0.51(0.21)	0.38 - 0.6	**0.04369**
GP32	1.31(0.53)	1.05 - 1.58	1.32(0.43)	1.14 - 1.57	0.51072
GP33	1.74(1.17)	1.21 - 2.39	1.82(1.42)	1.27 - 2.69	0.43979
GP34	0.34(0.08)	0.30 - 0.39	0.37(0.10)	0.33 - 0.43	**0.01096**
GP35	0.23(0.11)	0.17 - 0.29	0.25(0.15)	0.18 - 0.34	0.05454
GP36	0.41(0.09)	0.37 - 0.46	0.42(0.08)	0.39 - 0.48	**0.04733**
GP37	0.52(0.21)	0.40 - 0.61	0.51(0.24)	0.42 - 0.66	0.33962
GP38	0.86(0.26)	0.73 - 0.99	0.92(0.25)	0.79 - 1.04	**0.04925**
GP39	0.47(0.21)	0.40 - 0.61	0.52(0.26)	0.42 - 0.69	0.05781

Supplementary Table 5.3 Distribution of derived plasma N-glycan traits in Normal and MetS

	Normal		MetS		
N-glycan	Median(IQR)	Range	Median(IQR)	Range	p-value
Branching					
LB	82.97(4.33)	80.80 - 85.13	81.96(3.24)	80.51 - 83.76	0.006
HB	16.68(4.03)	14.45 - 18.49	17.84(3.39)	15.74 - 19.13	0.004
Level of sialylation					
S0	24.97(5.92)	22.16 - 28.09	24.79(6.23)	21.29 - 27.53	0.685
S1	20.87(1.95)	20.05 - 22	20.25(1.62)	19.48 - 21.1	0.004
S2	39.75(4.06)	37.57 - 41.63	39.99(3.81)	37.82 - 41.64	0.591
S3	11.09(2.77)	9.67 - 12.45	11.90(2.35)	10.36 - 12.72	0.011
S4	2.29(0.64)	2.02 - 2.66	2.46(0.59)	2.12 - 2.72	0.013
Level of galactosylation					
G0	9.23(3.62)	7.58 - 11.2	9.70(2.96)	8.28 - 11.25	0.155
G1	10.77(2.59)	9.35 - 11.95	10.60(2.69)	9.21 - 11.91	0.71
G2	62.81(5.71)	59.98 - 65.69	61.61(4.14)	59.47 - 63.62	0.017
G3	11.97(3.52)	10.07 - 13.59	12.81(3.87)	11.07 - 14.94	0.001
G4	4.44(1.40)	3.87 - 5.28	4.36(1.96)	3.63 - 5.59	0.606
Position of fucose					
FUC_A	1.99(0.66)	1.70 - 2.37	2.25(0.66)	1.99 - 2.65	0
FUC_C	38.29(7.13)	34.99 - 42.13	39.06(6.03)	35.27 - 41.3	0.783
Level of sialylation of biantennary glycans					
A2	8.23(3.50)	6.64 - 10.14	8.76(2.92)	7.21 - 10.13	0.17
A2G	72.30(4.50)	69.97 - 74.48	70.98(3.73)	69.16 - 72.9	0.001
BAMS	20.87(1.95)	20.05 - 22	20.25(1.62)	19.48 - 21.1	0.004
BADS	35.58(3.83)	33.63 - 37.47	35.60(3.62)	33.53 - 37.16	0.94
Degree of branching					
BA	80.75(4.23)	78.81 - 83.04	79.78(3.57)	78.09 - 81.67	0.003
TRIA	12.90(3.61)	10.92 - 14.53	13.79(3.82)	11.95 - 15.77	0
TA	4.71(1.49)	4.06 - 5.56	4.60(2.05)	3.85 - 5.9	0.622
DG9index	0.29(0.04)	0.27 - 0.32	0.31(0.05)	0.29 - 0.35	0

Supplementary Table 5.4 Correlation between derived plasma N-glycan traits and liver function markers

	Albumin			Globulin			ALP			GAMMAGT			DirectBil		
Peaks	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
Branching															
LB	-0.01	0.9322	0.932	0.11	0.0829	0.1203	-0.22	**0.0005**	**0.0013**	-0.129	0.0440	0.0660	0.144	0.0248	0.0702
HB	0.01	0.9012	0.932	-0.12	0.0535	0.0922	0.22	**0.0005**	**0.0013**	0.138	0.0311	0.0502	-0.145	0.0237	0.0702
Level of sialylation															
S0	-0.14	0.0256	0.134	0.23	0.0003	0.0013	-0.05	0.407	0.4498	-0.154	0.0165	0.0346	0.019	0.7676	0.8060
S1	0.07	0.2858	0.500	-0.11	0.0974	0.1203	-0.23	**0.0004**	**0.0013**	-0.16	0.0127	0.0346	0.219	0.0006	0.0040
S2	0.18	0.0062	0.055	-0.18	0.0045	0.0158	0.05	0.4637	0.4869	0.195	0.0023	0.0346	-0.04	0.5383	0.6650
S3	0.03	0.6249	0.820	-0.08	0.1988	0.2319	0.24	**0.0002**	**0.0013**	0.147	0.0221	0.0422	-0.13	0.0421	0.0750
S4	-0.08	0.2240	0.470	-0.11	0.087	0.1203	0.14	**0.0246**	**0.0414**	0.024	0.7085	0.7440	-0.122	0.0579	0.0875
Level of galactosylation															
G0	-0.12	0.0675	0.177	0.31	0	0.0000	0.12	0.0539	0.0809	-0.04	0.5397	0.6093	-0.136	0.0340	0.0714
G1	-0.12	0.0580	0.177	0.18	0.0056	0.0168	-0.07	0.2566	0.2994	-0.161	0.0119	0.0346	0.073	0.2567	0.3369
G2	0.17	0.0078	0.055	-0.23	0.0003	0.0013	-0.18	**0.0045**	**0.0086**	0.008	0.8989	0.8989	0.166	0.0094	0.0492
G3	0.03	0.5948	0.820	-0.12	0.0536	0.0922	0.2	**0.0015**	**0.0032**	0.156	0.0151	0.0346	-0.13	0.0428	0.0750
G4	-0.01	0.8387	0.932	-0.04	0.5041	0.5572	0.1	0.1383	0.1815	0.056	0.3857	0.4764	-0.03	0.6364	0.7034
A2	-0.12	0.0630	0.177	0.32	0	0.0000	0.12	0.06	0.0840	-0.038	0.5513	0.6093	-0.139	0.0302	0.0704
A2G	0.11	0.1020	0.238	-0.16	0.0159	0.0371	-0.27	0.0000	**0.0000**	-0.101	0.1148	0.1607	0.244	0.0001	0.0025
Position of fucose															
FUC_A	-0.05	0.4300	0.645	0.03	0.6533	0.6533	0.24	0.0001	**0.0011**	0.159	0.0132	0.0346	-0.15	0.0196	0.0702
FUC_C	-0.13	0.0504	0.177	0.24	0.0002	0.0013	-0.14	0.0256	**0.0414**	-0.175	0.0063	0.0346	0.082	0.2027	0.2837
Level of sialylation of biantennary glycans															
BAMS	0.07	0.2858	0.500	-0.11	0.0974	0.1203	-0.23	0.0004	**0.0013**	-0.16	0.0127	0.0346	0.219	0.0006	0.0040
BADS	0.17	0.0073	0.055	-0.17	0.007	0.0184	0.01	0.8938	0.8938	0.18	0.0050	0.0346	-0.016	0.8070	0.8070
Degree of branching															
BA	-0.02	0.7381	0.912	0.12	0.0571	0.0922	-0.23	0.0003	**0.0013**	-0.14	0.0290	0.0502	0.142	0.0268	0.0702
TRIA	0.05	0.4170	0.645	-0.12	0.053	0.0922	0.21	0.0009	**0.0021**	0.163	0.0110	0.0346	-0.122	0.0583	0.0875
TA	-0.01	0.8457	0.932	-0.03	0.6314	0.6533	0.09	0.1521	0.1879	0.059	0.3560	0.4672	-0.031	0.6301	0.7034

Supplementary Table 5.5 Correlations between derived plasma N-glycan traits and SBP, DBP, TC, TG, LDL-c, VLDL-c and HDL-c

	SBP			DBP			TC			TG			LDL-C			VLDL-C			HDL-C		
	rs	p		rs	p		rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
Level of branching																					
LB	-0.0378	0.544	0.709	-0.13	0.038	0.078	0.14	0.028	0.071	0.14	0.025	0.065	-0.13	0.044	0.065	-0.21	0.001	0.002	0.13	0.036	0.157
HB	0.0373	0.548	0.709	0.13	0.036	0.078	-0.16	0.015	0.067	-0.15	0.024	0.065	0.14	0.031	0.049	0.23	0.000	0.001	-0.14	0.025	0.157
S0	-0.0216	0.728	0.728	-0.02	0.791	0.870	-0.01	0.858	0.899	0.02	0.768	0.804									
Level of sialylation													-0.15	0.016	0.033	-0.10	0.111	0.144	0.03	0.635	0.986
S1	-0.108	0.082	0.200	-0.14	0.019	0.074	0.21	0.001	0.008	0.22	0.001	0.004	-0.16	0.013	0.032	-0.17	0.008	0.016	-0.02	0.806	0.986
S2	0.0222	0.722	0.728	-0.01	0.895	0.895	0.00	0.957	0.957	-0.04	0.538	0.658	0.19	0.002	0.025	0.12	0.073	0.113	0.00	0.997	1.000
S3	0.0604	0.331	0.607	0.13	0.039	0.078	-0.13	0.036	0.071	-0.13	0.042	0.072	0.15	0.022	0.040	0.22	0.000	0.001	-0.11	0.081	0.254
S4	0.0923	0.137	0.274	0.16	0.009	0.065	-0.10	0.113	0.177	-0.12	0.058	0.086	0.02	0.709	0.742	0.11	0.102	0.141	-0.09	0.184	0.404
Level of galactosylation																					
G0	0.1616	0.009	0.073	0.11	0.089	0.142	-0.14	0.027	0.071	-0.14	0.034	0.068	-0.04	0.540	0.606	0.06	0.390	0.451	0.09	0.170	0.404
G1	-0.0465	0.454	0.709	-0.01	0.858	0.895	0.03	0.659	0.740	0.07	0.257	0.332	-0.16	0.012	0.032	-0.12	0.066	0.111	0.00	0.989	1.000
G2	-0.1038	0.094	0.207	-0.16	0.008	0.065	0.20	0.002	0.011	0.17	0.009	0.042	0.01	0.899	0.899	-0.11	0.077	0.113	0.02	0.796	0.986
G3	-0.0279	0.654	0.728	0.10	0.099	0.145	-0.14	0.029	0.071	-0.13	0.043	0.072	0.16	0.015	0.033	0.25	0.000	0.001	-0.18	0.005	0.061
G4	0.1482	0.017	0.073	0.05	0.389	0.504	-0.03	0.672	0.740	-0.03	0.636	0.700	0.06	0.386	0.471	0.03	0.628	0.628	0.00	1.000	1.000
A2	0.1686	0.006	0.073	0.11	0.086	0.142	-0.15	0.023	0.071	-0.14	0.030	0.066	-0.04	0.551	0.606	0.05	0.423	0.465	0.09	0.149	0.404
A2G	-0.1339	0.031	0.096	-0.18	0.003	0.065	0.24	0.000	0.004	0.24	0.000	0.003	-0.10	0.115	0.158	-0.22	0.000	0.001	0.03	0.690	0.986
Position of fucose																					
FUC_A	0.1494	0.016	0.073	0.14	0.021	0.074	-0.12	0.056	0.095	-0.15	0.020	0.065	0.16	0.013	0.032	0.28	0.000	0.000	-0.04	0.513	0.940
FUC_C	-0.0264	0.671	0.728	-0.03	0.668	0.817	0.07	0.257	0.354	0.08	0.203	0.279	-0.17	0.006	0.032	-0.14	0.032	0.059	0.08	0.211	0.421
Level of sialylation of biantennary glycans																					
BAMS	-0.108	0.082	0.200	-0.14	0.019	0.074	0.21	0.001	0.008	0.22	0.001	0.004	-0.16	0.013	0.032	-0.17	0.008	0.016	-0.02	0.806	0.986
BADS	0.0435	0.484	0.709	-0.02	0.727	0.842	0.03	0.591	0.740	-0.02	0.807	0.807	0.18	0.005	0.032	0.06	0.377	0.451	0.04	0.555	0.940
Degree of branching																					
BA	-0.0381	0.540	0.709	-0.13	0.037	0.078	0.14	0.034	0.071	0.14	0.027	0.065	-0.14	0.029	0.049	-0.24	0.000	0.001	0.14	0.035	0.157
TRIA	-0.0266	0.669	0.728	0.10	0.091	0.142	-0.13	0.050	0.091	-0.12	0.058	0.086	0.16	0.011	0.032	0.27	0.000	0.000	-0.18	0.006	0.061
TA	0.1543	0.013	0.073	0.06	0.363	0.499	-0.03	0.661	0.740	-0.03	0.630	0.700	0.06	0.356	0.461	0.03	0.602	0.628	0.01	0.938	1.000

Supplementary Table 5.6 Correlations between derived plasma N-glycan traits and Age, WHtR, BMI and FPG

	Age			WHtR			BMI			FPG		
Peaks	rs	p	q	rs	q	q	rs	p	q	rs	p	q
Level of branching												
LB	-0.10	0.127	0.225	-0.26	0.000	3E-05	-0.22	0.000	0.001	-0.01	0.854	0.904
HB	0.10	0.127	0.225	0.29	0.000	3E-05	0.25	0.000	0.000	0.01	0.825	0.904
Level of sialylation												
S0	-0.03	0.612	0.612	-0.11	0.075	1E-01	-0.11	0.087	0.137	-0.03	0.591	0.904
S1	-0.12	0.051	0.153	-0.20	0.001	2E-03	-0.16	0.009	0.017	-0.09	0.164	0.402
S2	0.03	0.584	0.612	0.09	0.153	2E-01	0.10	0.110	0.151	0.11	0.084	0.402
S3	0.11	0.090	0.209	0.28	0.000	3E-05	0.23	0.000	0.001	0.03	0.586	0.904
S4	0.06	0.364	0.425	0.21	0.001	2E-03	0.19	0.002	0.005	-0.13	0.045	0.328
Level of galactosylation												
G0	0.30	0.000	0.000	-0.04	0.553	6E-01	-0.15	0.013	0.022	0.09	0.131	0.402
G1	-0.11	0.069	0.182	-0.08	0.193	3E-01	-0.04	0.545	0.601	-0.08	0.207	0.456
G2	-0.17	0.006	0.031	-0.12	0.059	1E-01	-0.04	0.546	0.601	-0.01	0.904	0.904
G3	0.06	0.321	0.421	0.33	0.000	3E-05	0.29	0.000	0.000	0.02	0.766	0.904
G4	0.09	0.160	0.240	0.02	0.788	8E-01	0.02	0.713	0.713	0.01	0.882	0.904
A2	0.30	0.000	0.000	-0.04	0.484	6E-01	-0.16	0.009	0.017	0.10	0.128	0.402
A2G	-0.28	0.000	0.000	-0.22	0.000	8E-04	-0.10	0.100	0.147	-0.07	0.248	0.497
Position of fucose												
FUC_A	0.15	0.013	0.053	0.35	0.000	3E-05	0.34	0.000	0.000	0.13	0.043	0.328
FUC_C	-0.08	0.195	0.272	-0.11	0.073	1E-01	-0.07	0.232	0.300	-0.05	0.384	0.705
Level of sialylation of biantennary glycans												
BAMS	-0.12	0.051	0.153	-0.20	0.001	2E-03	-0.16	0.009	0.017	-0.09	0.164	0.402
BADS	0.03	0.605	0.612	0.03	0.687	8E-01	0.04	0.502	0.601	0.10	0.126	0.402
Degree of branching												
BA	-0.09	0.139	0.225	-0.29	0.000	3E-05	-0.25	0.000	0.000	-0.02	0.753	0.904
TRIA	0.06	0.344	0.425	0.35	0.000	3E-05	0.31	0.000	0.000	0.03	0.653	0.904
TA	0.09	0.137	0.225	0.02	0.789	8E-01	0.02	0.707	0.713	0.02	0.752	0.904

Supplementary Table 5.7 Correlation between N-glycans and albumin, globulin, alkaline phosphatase

	Albumin		Globulin		Total protein		ALP	
Peaks	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**
GP1	-0.136	**0.03393**	0.319	**<0.00001**	0.194	**0.00233**	0.128	0.04587
GP2	-0.032	0.62182	0.209	**0.00103**	0.104	0.1073	0.047	0.46726
GP3	0.036	0.57499	0.031	0.63465	-0.005	0.93833	-0.066	0.30732
GP4	-0.135	**0.03493**	0.169	**0.00837**	0.068	0.29436	-0.087	0.17775
GP5	-0.110	0.08598	0.111	0.08342	0.034	0.60055	-0.025	0.69565
GP6	-0.119	0.06292	0.190	**0.00295**	0.053	0.41206	-0.010	0.87865
GP7	0.034	0.597	-0.050	0.43579	-0.038	0.55667	0.057	0.37985
GP8	0.081	0.20597	-0.188	0.00325	-0.115	0.07305	-0.170	**0.00779**
GP9	-0.006	0.92059	0.014	0.8265	-0.015	0.81034	-0.103	0.10937
GP10	-0.031	0.63183	-0.104	0.10657	-0.087	0.17665	-0.295	**<0.00001**
GP11	-0.083	0.19701	0.098	0.12834	0.055	0.39141	-0.117	0.06753
GP12	0.159	**0.01271**	-0.150	**0.01901**	-0.065	0.31642	-0.111	0.08428
GP13	-0.125	0.05104	0.187	**0.00346**	0.090	0.16077	-0.024	0.71099
GP14	0.098	0.12543	-0.176	**0.00591**	-0.089	0.16598	0.013	0.84135
GP15	-0.026	0.68608	0.088	0.17318	0.054	0.40036	-0.060	0.35328
GP16	0.009	0.88663	-0.031	0.63521	0.010	0.87949	-0.324	**<0.00001**
GP17	-0.108	0.09337	0.163	0.01075	0.082	0.20089	-0.009	0.88872
GP18	0.170	**0.00769**	-0.207	**0.00115**	-0.112	0.08264	-0.118	0.06547
GP19	0.253	**0.00006**	-0.106	0.10006	0.001	0.9825	0.250	**0.00008**
GP20	0.173	**0.00667**	-0.185	0.00372	-0.072	0.26322	0.100	0.12144
GP21	0.057	0.37754	0.010	0.88195	-0.001	0.98661	-0.044	0.49722
GP22	0.096	0.13516	0.062	0.33699	0.087	0.17746	-0.136	**0.03398**
GP23	-0.094	0.14357	0.200	**0.00175**	0.097	0.13178	-0.058	0.36499
GP24	0.019	0.77054	-0.212	**0.0009**	-0.129	**0.04494**	0.058	0.36962
GP25	0.015	0.8138	-0.111	0.08436	-0.108	0.09258	-0.185	**0.00383**
GP26	0.008	0.90384	-0.082	0.20126	-0.038	0.55367	0.192	**0.00271**
GP27	0.023	0.72362	0.038	0.55046	0.023	0.72377	0.015	0.81501
GP28	0.027	0.67334	-0.156	**0.01508**	-0.095	0.14163	0.076	0.23882
GP29	0.103	0.10942	-0.061	0.34493	-0.018	0.77546	-0.074	0.25352
GP30	0.046	0.47535	-0.116	0.07196	-0.039	0.54393	0.182	**0.00443**
GP31	0.030	0.63707	0.006	0.92574	0.027	0.6794	0.124	0.05265
GP32	-0.025	0.69786	0.023	0.72512	0.018	0.78094	0.321	**<0.00001**
GP33	0.003	0.95726	0.036	0.58158	0.017	0.78946	0.030	0.64158
GP34	-0.041	0.51901	0.006	0.9253	-0.025	0.69981	0.190	**0.00299**
GP35	0.004	0.95309	0.074	0.25129	0.039	0.54206	0.039	0.54616
GP36	-0.074	0.25129	-0.168	**0.00863**	-0.183	**0.0042**	0.155	**0.01589**
GP37	-0.043	0.50406	-0.119	0.06293	-0.104	0.10702	0.092	0.15506
GP38	-0.069	0.28655	-0.076	0.23678	-0.093	0.14731	0.174	**0.00647**
GP39	-0.029	0.6487	-0.032	0.61824	-0.057	0.37957	0.057	0.37573

Supplementary Table 5.8 Correlation between derived traits and albumin, globulin, total protein, ALP

	Albumin		Globulin		Total protein		ALP	
Peaks	**rs**	p	**rs**	p	**rs**	p	**rs**	p
Branching								
LB	-0.005	0.93222	0.111	0.08287	0.082	0.20397	-0.220	**0.00054**
HB	0.008	0.90123	-0.124	0.0535	-0.092	0.15406	0.220	**0.00054**
Level of sialylation								
S0	-0.143	**0.02557**	0.229	**0.00032**	0.108	0.09299	-0.053	0.40702
S1	0.069	0.28579	-0.107	0.09742	-0.036	0.58098	-0.227	**0.00037**
S2	0.175	**0.00624**	-0.182	**0.0045**	-0.073	0.25718	0.047	0.46373
S3	0.031	0.6249	-0.083	0.1988	-0.051	0.42614	0.235	**0.00022**
S4	-0.078	0.224	-0.110	0.08702	-0.135	**0.03593**	0.144	**0.02459**
Level of galactosylation								
G0	-0.117	0.06746	0.310	**<0.00001**	0.183	**0.00416**	0.124	0.0539
G1	-0.122	0.05803	0.177	**0.00564**	0.076	0.23753	-0.073	0.2566
G2	0.170	**0.00775**	-0.231	**0.00028**	-0.101	0.11673	-0.182	**0.00453**
G3	0.034	0.59477	-0.124	0.05355	-0.059	0.36132	0.203	**0.0015**
G4	-0.013	0.83867	-0.043	0.50414	-0.051	0.42604	0.095	0.13827
A2	-0.119	0.06297	0.318	**<0.00001**	0.190	**0.00297**	0.121	0.06002
A2G	0.105	0.10198	-0.155	**0.01591**	-0.080	0.21486	-0.270	**0.00002**
Position of fucose								
FUC_A	-0.051	0.43	0.029	0.65326	-0.015	0.82106	0.242	**0.00014**
FUC_C	-0.125	0.05041	0.239	**0.00017**	0.130	**0.04371**	-0.143	**0.02556**
Level of sialylation of biantennary glycans								
BAMS	0.069	0.28579	-0.107	0.09742	-0.036	0.58098	-0.227	**0.00037**
BADS	0.172	**0.00725**	-0.173	**0.007**	-0.074	0.25253	0.009	0.89381
Degree of branching								
BA	-0.022	0.73812	0.122	0.0571	0.086	0.18286	-0.232	**0.00026**
TRIA	0.052	0.41702	-0.124	0.05299	-0.052	0.41752	0.211	**0.00092**
TA	-0.013	0.84572	-0.031	0.63144	-0.043	0.50389	0.092	0.1521
DG9ind	-0.033	0.6128	0.195	**0.00222**	0.140	**0.02878**	0.143	**0.02566**

Supplementary Table 5.9 Correlation between N-glycans and ALT/GPT, AST/GOT, GAMMAGT, direct and indirect bilirubin

	ALTGPT		ASTGOT		GAMMAGT		DBil		IndBil		TBil	
Peaks	**rs**	p	**rs**	p	**rs**	p	**rs**	p	**rs**	p	**rs**	
GP1	0.038	0.5541	0.076	0.24092	-0.038	0.55538	-0.160	0.01258	-0.185	**0.00382**	-0.175	**0.00633**
GP2	0.077	0.22899	0.109	0.08898	-0.043	0.50822	-0.008	0.90411	-0.005	0.93364	0.007	0.91364
GP3	0.066	0.30336	0.102	0.11225	-0.030	0.64103	0.102	0.1125	0.123	0.05607	0.134	**0.03756**
GP4	-0.030	0.64279	-0.047	0.46401	-0.160	**0.01234**	0.061	0.34727	-0.046	0.47551	0.015	0.81085
GP5	0.018	0.78107	0.027	0.67238	-0.138	**0.03162**	0.021	0.74968	-0.084	0.19374	-0.030	0.64671
GP6	-0.038	0.55242	-0.008	0.90249	-0.098	0.12903	0.087	0.1744	0.022	0.73339	0.058	0.36746
GP7	-0.009	0.89113	-0.020	0.75449	-0.005	0.93744	0.037	0.56507	0.070	0.27913	0.056	0.38663
GP8	-0.018	0.77435	0.020	0.75743	-0.047	0.46836	0.149	0.02012	0.099	0.12406	0.135	**0.03523**
GP9	-0.065	0.31558	0.065	0.31659	-0.107	0.09501	0.136	0.03437	0.157	0.01436	0.163	**0.01116**
GP10	-0.101	0.11722	-0.016	0.80473	-0.220	**0.00055**	0.230	0.00031	0.118	0.06709	0.177	**0.00567**
GP11	-0.148	**0.02139**	0.006	0.93184	-0.173	**0.00686**	0.125	0.05236	0.114	0.07722	0.112	0.08134
GP12	-0.071	0.27003	-0.051	0.43207	0.015	0.81134	0.100	0.12121	0.103	0.11034	0.107	0.09593
GP13	0.046	0.47165	0.098	0.12665	-0.128	**0.04669**	0.024	0.71424	-0.027	0.67163	-0.004	0.94714
GP14	-0.042	0.51854	-0.063	0.32766	0.041	0.52982	-0.015	0.81946	-0.031	0.62824	-0.031	0.63342
GP15	-0.133	**0.03845**	0.051	0.43307	-0.127	0.04725	0.091	0.15945	0.187	**0.00348**	0.149	**0.02010**
GP16	-0.095	0.13778	0.084	0.19405	-0.193	**0.00258**	0.236	0.00021	0.206	**0.00124**	0.226	**0.00038**
GP17	-0.141	**0.0280**	0.009	0.886	).132	.04041	).028	0.66	0.09	0.140	0.05	0.43
GP18	-0.013	0.83841	-0.064	0.32314	-0.014	0.82531	0.127	0.04803	0.077	0.23453	0.092	0.15100
GP19	0.219	**0.00059**	-0.002	0.971	0.273	**0.00002**	-0.004	0.94762	0.069	0.28578	0.041	0.52147
GP20	0.130	**0.04227**	-0.006	0.92152	0.227	**0.00035**	-0.045	0.48456	0.009	0.88789	-0.021	0.74451
GP21	0.091	0.15687	0.110	0.08814	-0.048	0.46003	0.038	0.5505	0.106	0.10084	0.078	0.22869
GP22	0.088	0.17353	0.129	0.04525	0.046	0.47148	0.087	0.17788	0.193	**0.00246**	0.155	**0.01539**
GP23	-0.036	0.57205	0.121	0.0591	-0.049	0.44601	0.057	0.37536	0.173	**0.00674**	0.131	**0.04161**
GP24	-0.125	0.05102	-0.248	0.00009	0.084	0.19281	-0.053	0.41415	-0.123	0.05579	-0.097	0.1328
GP25	-0.117	0.06892	-0.113	0.07978	-0.129	0.04496	0.080	0.21188	0.092	0.15471	0.093	0.1486
GP26	-0.044	0.49016	-0.173	0.00684	0.169	0.00815	-0.125	0.05201	-0.132	**0.03941**	-0.135	0.03534
GP27	0.093	0.14877	0.105	0.10221	0.050	0.43774	0.034	0.60104	0.017	0.78659	0.019	0.76675
GP28	-0.106	0.1002	-0.184	0.00392	0.065	0.31317	-0.031	0.63288	-0.054	0.40585	-0.051	0.43186
GP29	0.010	0.87278	0.056	0.38218	0.070	0.27783	0.053	0.4113	0.063	0.3289	0.051	0.42628
GP30	-0.057	0.37643	-0.174	0.00645	0.113	0.0796	-0.109	0.08888	-0.094	0.14213	-0.110	0.08851
GP31	-0.017	0.78901	-0.108	0.09434	0.159	**0.01297**	-0.179	0.00516	-0.111	0.0848	-0.150	**0.01897**
GP32	0.023	0.7156	-0.090	0.16411	0.212	**0.00087**	-0.182	0.00433	-0.122	0.05657	-0.155	**0.01558**
GP33	0.068	0.28982	0.090	0.16003	0.042	0.5134	0.030	0.64235	0.026	0.68297	0.023	0.72384
GP34	-0.051	0.42898	-0.101	0.11589	0.179	**0.00511**	-0.207	0.00117	-0.154	0.01594	-0.188	**0.00321**
GP35	0.064	0.32143	0.047	0.46554	0.074	0.25271	-0.045	0.48349	-0.034	0.59917	-0.049	0.44479
GP36	-0.123	0.0546	-0.153	0.01667	0.062	0.33635	-0.141	0.02796	-0.134	**0.0371**	-0.146	**0.02296**
GP37	-0.149	0.02006	-0.136	0.03369	-0.005	0.9383	-0.069	0.28735	-0.064	0.32398	-0.073	0.25641
GP38	-0.084	0.19061	-0.097	0.13308	0.057	0.37609	-0.160	0.01257	-0.087	0.17686	-0.130	**0.04335**
GP39	0.029	0.65197	0.022	0.73278	0.030	0.64133	-0.040	0.53796	0.004	0.95574	-0.023	0.71591

Supplementary Table 5.10 Correlation between derived N-glycans and ALT/GPT, AST/GOT, GAMMAGT, Direct and indirect bilirubin

	ALTGPT		ASTGOT		GAMMAGT		DirectBil		IndirectBil		TotalBil	
Peaks	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**
Branching												
LB	0.032	0.61427	0.135	**0.03579**	-0.129	**0.04401**	0.144	**0.02483**	0.114	0.07586	0.141	**0.02761**
HB	-0.048	0.45569	-0.144	**0.02471**	0.138	**0.03109**	-0.145	**0.02374**	-0.139	**0.03017**	-0.155	**0.01532**
Level of sialylation												
S0	-0.017	0.78751	0.031	0.63525	-0.154	**0.01648**	0.019	0.76764	-0.055	0.39028	-0.012	0.85833
S1	-0.186	**0.0036**	-0.016	0.80645	-0.160	**0.01268**	0.219	**0.00057**	0.201	**0.00168**	0.208	**0.00113**
S2	0.102	0.11199	0.000	0.99699	0.195	**0.00227**	-0.040	0.53831	0.048	0.45427	0.003	0.95721
S3	-0.010	0.87704	-0.112	0.08066	0.147	**0.02208**	-0.130	**0.04214**	-0.116	0.07185	-0.135	**0.03566**
S4	-0.108	0.09372	-0.086	0.18351	0.024	0.70854	-0.122	0.05794	-0.070	0.27967	-0.102	0.11262
Level of galactosylation												
G0	0.056	0.38828	0.088	0.16983	-0.040	0.53974	-0.136	**0.03398**	-0.150	**0.01912**	-0.142	**0.02724**
G1	-0.016	0.80266	0.001	0.98905	-0.161	**0.0119**	0.073	0.25672	-0.032	0.61429	0.028	0.65885
G2	-0.023	0.72094	0.017	0.79568	0.008	0.89894	0.166	**0.00937**	0.222	**0.00048**	0.198	**0.00192**
G3	-0.044	0.49357	-0.178	**0.00532**	0.156	**0.01511**	-0.130	**0.04283**	-0.130	**0.04213**	-0.140	**0.02897**
G4	-0.012	0.85463	0.000	0.99992	0.056	0.38567	-0.030	0.63641	-0.011	0.87005	-0.027	0.67236
A2	0.054	0.40071	0.093	0.14942	-0.038	0.55129	-0.139	**0.03016**	-0.155	**0.01542**	-0.146	**0.023**
A2G	-0.041	0.52173	0.026	0.69031	-0.101	0.11482	0.244	**0.00012**	0.220	**0.00056**	0.239	**0.00017**
Position of fucose												
FUC_A	0.041	0.52069	-0.042	0.51261	0.159	**0.01318**	-0.150	**0.01955**	-0.084	0.19328	-0.123	0.05614
FUC_C	-0.039	0.54993	0.081	0.21057	-0.175	**0.00625**	0.082	0.20265	0.044	0.49202	0.073	0.25736
Level of sialylation of biantennary glycans												
BAMS	-0.186	**0.0036**	-0.016	0.80645	-0.160	**0.01268**	0.219	**0.00057**	0.201	**0.00168**	0.208	**0.00113**
BADS	0.116	0.07004	0.038	0.5552	0.180	**0.00501**	-0.016	0.80695	0.084	0.19138	0.035	0.5913
Degree of branching												
BA	0.019	0.77302	0.135	**0.03493**	-0.140	**0.02896**	0.142	**0.02676**	0.106	0.09887	0.136	**0.0339**
TRIA	-0.031	0.63044	-0.177	**0.00555**	0.163	**0.01095**	-0.122	0.05833	-0.116	0.07177	-0.126	**0.04961**
TA	-0.004	0.94492	0.005	0.93815	0.059	0.35596	-0.031	0.63011	-0.011	0.86278	-0.028	0.66099
DG9ind	0.141	**0.02758**	0.126	**0.04929**	0.221	**0.00053**	-0.166	**0.00958**	-0.043	0.50002	-0.098	0.12622

Supplementary Table 5.11 Correlation between N-glycans and kidney function markers-creatinine, urea and uric acid

	Creatinine		Urea		Uric acid		CKD-risk	
Peaks	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**
GP1	-0.109	0.08953	0.029	0.6544	0.018	0.78082	0.011	0.86523
GP2	-0.023	0.72477	0.006	0.9216	-0.006	0.92811	-0.013	0.84295
GP3	0.053	0.40829	-0.039	0.5419	-0.058	0.36818	0.025	0.69545
GP4	-0.113	0.07935	0.020	0.7576	-0.132	**0.04001**	0.180	**0.00523**
GP5	-0.069	0.28723	0.040	0.5392	-0.048	0.45849	0.129	**0.04587**
GP6	-0.175	**0.00625**	-0.052	0.4197	-0.188	**0.00339**	0.069	0.28748
GP7	-0.052	0.42274	-0.044	0.4982	-0.046	0.47348	0.050	0.43899
GP8	0.140	**0.02954**	-0.019	0.7642	-0.080	0.21384	0.054	0.40261
GP9	-0.029	0.65591	-0.081	0.2072	-0.094	0.14773	0.117	0.07012
GP10	-0.035	0.58495	-0.075	0.2464	-0.170	**0.00808**	0.261	**0.00004**
GP11	-0.098	0.12858	-0.130	**0.043**	-0.177	**0.00593**	0.116	0.07195
GP12	0.024	0.70604	-0.010	0.8731	-0.049	0.45172	0.009	0.89116
GP13	-0.107	0.09755	-0.046	0.4811	0.003	0.96454	0.128	**0.0480**1
GP14	0.081	0.20811	0.030	0.6398	-0.026	0.6931	-0.125	0.05384
GP15	-0.102	0.11266	-0.077	0.2301	-0.034	0.6013	0.119	0.06535
GP16	-0.031	0.63441	-0.163	**0.011**	-0.050	0.43689	0.274	**0.00002**
GP17	-0.087	0.17979	-0.082	0.2031	-0.072	0.26671	0.024	0.70801
GP18	0.156	**0.01528**	0.071	0.2689	-0.028	0.66223	-0.063	0.32909
GP19	0.081	0.20991	0.051	0.4272	0.264	**0.00003**	-0.136	**0.03465**
GP20	0.159	**0.01303**	0.094	0.1445	0.113	0.0799	-0.205	**0.00144**
GP21	0.101	0.11676	0.050	0.437	0.136	**0.03426**	-0.087	0.17993
GP22	-0.008	0.90189	-0.117	0.0687	0.203	**0.00153**	0.173	**0.00736**
GP23	-0.128	**0.04651**	-0.089	0.1681	0.076	0.24123	0.114	0.07701
GP24	-0.040	0.53155	0.053	0.4162	-0.114	0.0778	-0.071	0.27501
GP25	0.005	0.93574	-0.027	0.6796	0.096	0.1358	0.018	0.77902
GP26	-0.133	**0.03829**	0.017	0.7922	-0.044	0.4965	-0.040	0.53917
GP27	0.204	**0.00141**	0.025	0.7015	0.107	0.09792	-0.177	**0.006**
GP28	-0.050	0.44328	0.007	0.9095	-0.066	0.30398	-0.001	0.98196
GP29	0.086	0.18313	-0.024	0.7159	0.018	0.78209	-0.008	0.90031
GP30	-0.074	0.25077	0.038	0.56	-0.027	0.67771	-0.050	0.43868
GP31	-0.194	**0.00243**	-0.072	0.2617	0.004	0.95672	0.030	0.6405
GP32	-0.094	0.14655	0.055	0.3908	0.045	0.48746	-0.122	0.05974
GP33	0.203	**0.00153**	0.024	0.7052	0.104	0.10628	-0.180	**0.00507**
GP34	-0.196	**0.0022**	-0.048	0.4598	0.026	0.68472	-0.030	0.64922
GP35	0.117	0.06893	-0.039	0.5485	0.095	0.14038	-0.165	**0.01034**
GP36	-0.069	0.28334	-0.018	0.7804	-0.021	0.74265	-0.171	**0.00796**
GP37	-0.092	0.15463	-0.042	0.5192	-0.093	0.15215	0.018	0.77753
GP38	-0.078	0.22628	-0.061	0.3446	-0.030	0.6482	-0.086	0.18312
GP39	0.122	0.05781	-0.017	0.7927	0.079	0.22152	-0.186	**0.00375**

Supplementary Table 5.12 Correlation between derived N-glycans traits and kidney function markers-creatinine, urea and uric acid

	Creatinine		Urea		Uric acid		CKD-risk	
Peaks	**rs**	**p**	**rs**	**p**	**rs**	**p**	**rs**	**p**
Branching								
LB	-0.013	0.83741	-0.018	0.775	-0.031	0.63757	0.182	**0.00468**
HB	-0.003	0.96217	0.037	0.5636	0.023	0.72537	-0.18	**0.00474**
Level of sialylation								
S0	-0.116	0.07231	-0.007	0.915	-0.117	0.07068	0.154	**0.0167**
S1	0.036	0.57552	-0.082	0.2016	-0.049	0.44462	0.083	0.20019
S2	0.109	0.09165	0.044	0.5001	0.149	**0.02059**	-0.13	**0.0448**
S3	0.027	0.67576	0.047	0.467	0.059	0.359	-0.2	**0.00233**
S4	-0.025	0.70092	-0.057	0.3735	-0.021	0.74547	-0.13	**0.03801**
Level of galactosylation								
G0	-0.096	0.13537	0.027	0.6789	0.014	0.82538	0.011	0.86424
G1	-0.116	0.0718	0.015	0.8156	-0.118	0.06742	0.169	**0.00872**
G2	0.132	**0.04068**	-0.046	0.4753	0.059	0.36235	0.013	0.83586
G3	-0.062	0.33506	0.045	0.4825	-0.014	0.82355	-0.1	0.14102
G4	0.153	**0.01712**	-0.016	0.8044	0.082	0.20681	-0.21	**0.00114**
A2	-0.095	0.13911	0.030	0.6394	0.024	0.71532	0.01	0.87318
A2G	0.072	0.26174	-0.048	0.4544	-0.040	0.54001	0.131	**0.04203**
Position of fucose								
FUC_A	-0.005	0.93491	0.032	0.6203	0.107	0.09733	-0.2	**0.00172**
FUC_C	-0.136	**0.03513**	-0.075	0.2478	-0.061	0.34776	0.241	**0.00016**
Level of sialylation of biantennary glycans								
BAMS	0.036	0.57552	-0.082	0.2016	-0.049	0.44462	0.083	0.20019
BADS	0.146	**0.02351**	0.038	0.5522	0.162	**0.01174**	-0.13	**0.03867**
Degree of branching								
BA	-0.011	0.85923	-0.022	0.7346	-0.041	0.53152	0.184	**0.00422**
TRIA	-0.064	0.31973	0.052	0.418	0.001	0.98578	-0.09	0.15883
TA	0.152	**0.01817**	-0.017	0.7963	0.087	0.17955	-0.21	**0.00111**
DG9ind	-0.089	0.16955	-0.042	0.5147	0.120	0.06199	-0.05	0.47177

Appendix II

Chapter Six Supplementary Data

Supplementary Table 6.2 Correlations between individual plasma N-glycan peaks and SBP and DBP

	SBP						DBP					
	CONTROLS			CASES			CONTROLS			CASES		
Peak	rs	p	q	rs	p	q	rs	p	q	rs	p	q
GP1	0.14	0.0383	0.1949	0.06	0.3729	0.8039	0.12	0.0784	0.3666	0.07	0.2881	0.9149
GP2	0.12	0.0856	0.3073	0.13	0.0438	0.4308	0.05	0.5063	0.7354	0.06	0.3411	0.9149
GP3	0.04	0.5863	0.9018	0.16	0.0145	0.4156	-0.06	0.3565	0.6214	0.07	0.3191	0.9149
GP4	-0.06	0.3411	0.7174	-0.15	0.0204	0.4156	-0.01	0.8573	0.9338	-0.09	0.1784	0.9149
GP5	-0.04	0.5389	0.9018	-0.09	0.1998	0.7248	-0.01	0.9213	0.9646	-0.03	0.6672	0.9149
GP6	0.01	0.9090	0.9377	0.05	0.4086	0.8039	0.04	0.5571	0.7388	-0.03	0.6853	0.9149
GP7	-0.08	0.2478	0.6820	0.04	0.5435	0.8961	0.00	0.9733	0.9733	-0.03	0.6147	0.9149
GP8	0.01	0.8867	0.9377	0.05	0.4257	0.8114	-0.11	0.1053	0.3666	0.01	0.8584	0.9533
GP9	-0.02	0.7673	0.9327	0.14	0.0294	0.4308	-0.06	0.3466	0.6214	0.05	0.4343	0.9149
GP10	-0.21	0.0018	0.1086	-0.19	0.0032	0.1940	-0.17	0.0140	0.3666	-0.12	0.0691	0.9149
GP11	-0.12	0.0841	0.3073	0.01	0.8394	0.9562	-0.05	0.4518	0.7017	-0.06	0.3965	0.9149
GP12	-0.01	0.8766	0.9377	-0.02	0.7633	0.9502	-0.08	0.2675	0.6214	-0.02	0.7248	0.9149
GP13	0.02	0.7219	0.9327	0.04	0.5381	0.8961	0.05	0.4601	0.7017	0.04	0.5579	0.9149
GP14	0.01	0.8649	0.9377	-0.01	0.9195	0.9562	-0.07	0.3229	0.6214	-0.06	0.3362	0.9149
GP15	0.03	0.6218	0.9018	0.11	0.1080	0.6590	0.06	0.3787	0.6418	0.04	0.5946	0.9149
GP16	-0.17	0.0134	0.1949	-0.09	0.1575	0.7248	-0.11	0.0925	0.3666	-0.07	0.2681	0.9149
GP17	0	0.9786	0.9786	0.06	0.3835	0.8039	0.07	0.3335	0.6214	-0.02	0.7349	0.9149
GP18	-0.04	0.5372	0.9018	-0.09	0.1915	0.7248	-0.08	0.2638	0.6214	-0.07	0.2644	0.9149
GP19	0.04	0.5116	0.9018	0.07	0.2799	0.7248	0.03	0.6889	0.8576	0.09	0.1602	0.9149
GP20	0.01	0.9224	0.9377	0.08	0.2385	0.7248	-0.02	0.7276	0.8702	0.04	0.5627	0.9149
GP21	0.03	0.6089	0.9018	0.11	0.0844	0.5754	0.02	0.8243	0.9301	0.03	0.6800	0.9149
GP22	0.07	0.2950	0.6820	0.02	0.7200	0.9502	0.02	0.7238	0.8702	0.01	0.8841	0.9533
GP23	0.14	0.0430	0.2016	0.13	0.0451	0.4308	0.06	0.3902	0.6433	0.04	0.5867	0.9149
GP24	-0.09	0.1701	0.5462	-0.08	0.2398	0.7248	-0.02	0.7766	0.8938	-0.03	0.6263	0.9149
GP25	-0.02	0.7983	0.9327	0.02	0.7439	0.9502	-0.13	0.0532	0.3666	-0.12	0.0747	0.9149
GP26	-0.03	0.6505	0.9018	-0.03	0.6413	0.9159	0.04	0.5478	0.7388	0.02	0.7092	0.9149
GP27	0.15	0.0309	0.1949	-0.01	0.8369	0.9562	0.01	0.8386	0.9301	-0.06	0.3310	0.9149
GP28	-0.07	0.2917	0.6820	-0.06	0.3403	0.7785	0.03	0.6306	0.8014	-0.01	0.8780	0.9533
GP29	0.02	0.7819	0.9327	-0.11	0.0849	0.5754	0.04	0.5328	0.7388	-0.09	0.1747	0.9149
GP30	-0.07	0.3122	0.6820	-0.03	0.6202	0.9159	0.05	0.4508	0.7017	0.04	0.5130	0.9149
GP31	0.01	0.8688	0.9377	-0.03	0.6045	0.9159	0.12	0.0874	0.3666	0.01	0.9285	0.9533
GP32	0.03	0.6247	0.9018	0.06	0.3446	0.7785	0.11	0.1142	0.3666	0.08	0.2325	0.9149
GP33	0.14	0.0328	0.1949	0.01	0.9149	0.9562	0.02	0.7461	0.8753	-0.05	0.4589	0.9149
GP34	0.06	0.3675	0.7472	-0.02	0.7623	0.9502	0.15	0.0269	0.3666	-0.01	0.9377	0.9533
GP35	0.19	0.0045	0.1363	0.01	0.8259	0.9562	0.08	0.2162	0.5735	-0.05	0.4237	0.9149
GP36	0.02	0.7365	0.9327	-0.08	0.2340	0.7248	0.08	0.2490	0.6214	-0.09	0.1794	0.9149
GP37	-0.07	0.3131	0.6820	-0.07	0.2767	0.7248	0.07	0.2828	0.6214	0.00	0.9553	0.9553
GP38	0.02	0.7515	0.9327	-0.06	0.4007	0.8039	0.14	0.0418	0.3666	-0.01	0.8610	0.9533
GP39	0.15	0.0244	0.1949	0.00	0.9712	0.9874	0.05	0.4817	0.7167	-0.06	0.4019	0.9149

Supplementary Table 6.3 Correlation between derived plasma N-glycan traits and SBP and DBP in T2DM and controls

	SBP						DBP					
	CONTROLS			CASES			CONTROLS			CASES		
	rs	p	q	rs	p	q	rs	p	q	rs	p	q
LB	-0.05	0.4443	0.8743	0.04	0.5004	0.8961	-0.11	0.1019	0.3666	0.01	0.8798	0.95332
HB	0.04	0.5571	0.9018	-0.03	0.6623	0.9182	0.1	0.1285	0.3717	0.01	0.8966	0.95332
S0	-0.02	0.8104	0.9327	-0.05	0.4979	0.8961	0.01	0.9174	0.9646	-0.01	0.9170	0.95332
S1	-0.08	0.2683	0.6820	-0.01	0.9249	0.9562	-0.1	0.1341	0.3717	-0.08	0.2146	0.91491
S2	0.01	0.9026	0.9377	0.07	0.2737	0.7248	-0.03	0.6248	0.8014	0.04	0.5539	0.91491
S3	0.07	0.3037	0.6820	0	0.9993	0.9993	0.11	0.0976	0.3666	0.02	0.7194	0.91491
S4	0.08	0.2341	0.6820	-0.07	0.3160	0.7709	0.14	0.0428	0.3666	-0.06	0.4012	0.91491
G0	0.14	0.0364	0.1949	0.08	0.2467	0.7248	0.11	0.0922	0.3666	0.07	0.3059	0.91491
G1	-0.04	0.5678	0.9018	-0.1	0.1240	0.6877	0	0.9674	0.9733	-0.07	0.3233	0.91491
G2	-0.09	0.1642	0.5462	0.04	0.5962	0.9159	-0.15	0.0272	0.3666	-0.03	0.7023	0.91491
G3	-0.03	0.6484	0.9018	-0.03	0.6195	0.9159	0.07	0.3350	0.6214	0.03	0.6148	0.91491
G4	0.15	0.0313	0.1949	-0.01	0.8528	0.9562	0.06	0.3426	0.6214	-0.06	0.3403	0.91491
FUC_A	0.15	0.0254	0.1949	0.07	0.2852	0.7248	0.12	0.0884	0.3666	0.03	0.6407	0.91491
FUC_C	-0.02	0.7368	0.9327	-0.02	0.7959	0.9562	0.01	0.9330	0.9646	-0.01	0.8614	0.95332
BA	-0.05	0.4804	0.9018	0.04	0.5422	0.8961	-0.11	0.1092	0.3666	0.01	0.9060	0.95332
A2	0.15	0.0289	0.1949	0.07	0.2774	0.7248	0.11	0.0911	0.3666	0.07	0.3120	0.91491
A2G	-0.12	0.0737	0.2998	-0.02	0.7504	0.9502	-0.16	0.0177	0.3666	-0.07	0.3279	0.91491
BAMS	-0.08	0.2683	0.6820	-0.01	0.9249	0.9562	-0.1	0.1341	0.3717	-0.08	0.2146	0.91491
BADS	0.03	0.7091	0.9327	0.09	0.1950	0.7248	-0.04	0.5459	0.7388	0.03	0.6899	0.91491
TRIA	-0.03	0.6263	0.9018	-0.03	0.6457	0.9159	0.06	0.3453	0.6214	0.04	0.5967	0.91491
TA	0.15	0.0247	0.1949	-0.01	0.8806	0.9562	0.07	0.3103	0.6214	-0.06	0.3534	0.91491
DG9ind	0.13	0.0527	0.2296	0.13	0.0494	0.4308	0.12	0.0866	0.3666	0.11	0.0981	0.91491

Supplementary Table 6.4 Correlation between individual plasma N-glycan peaks and Age, HDL-c and BMI

	Age						HDL-c						BMI					
	Control			Case			Control			Case			CONTROLS			CASES		
	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
GP1	0.18	0.0065	0.0393	0.14	0.0396	0.1725	0.06	0.4192	0.7307	0	0.9646	0.9932	-0.13	0.05339	0.098691	-0.02	0.72042	0.836186
GP2	0.21	0.0016	0.0207	0.26	0.0001	0.0037	0.06	0.3981	0.7307	-0.06	0.3699	0.6838	-0.27	0.00005	0.000218	-0.21	0.0018	0.00732
GP3	0.07	0.3298	0.4907	0.22	0.0008	0.0235	-0.07	0.3337	0.7307	-0.13	0.0587	0.5083	-0.29	0.00002	0.000122	-0.28	0.00002	0.000174
GP4	-0.2	0.0027	0.0234	-0.03	0.6329	0.757	-0.05	0.5004	0.8033	-0.1	0.1339	0.5196	-0.06	0.38886	0.494176	0.03	0.60644	0.787082
GP5	-0.17	0.0107	0.0592	0.02	0.7658	0.865	-0.04	0.6126	0.8897	-0.08	0.2158	0.5459	0.01	0.86935	0.914316	0.06	0.38667	0.55049
GP6	0.06	0.399	0.5531	0.17	0.0098	0.0882	0.08	0.2565	0.6847	-0.08	0.2237	0.5459	-0.04	0.57052	0.656636	0.03	0.65697	0.834899
GP7	-0.06	0.372	0.5403	0.01	0.9383	0.985	0.05	0.5183	0.8107	-0.01	0.8715	0.987	0.18	0.00783	0.019901	0.01	0.85159	0.911351
GP8	-0.1	0.1364	0.3382	0.1	0.1168	0.2639	-0.12	0.1012	0.5476	-0.14	0.0431	0.5083	-0.3	0.00001	6.78E-05	-0.27	0.00004	0.000305
GP9	0.03	0.6761	0.7499	0.14	0.0301	0.1534	-0.03	0.7227	0.9248	-0.1	0.1354	0.5196	-0.27	0.00007	0.000285	-0.28	0.00002	0.000174
GP10	-0.41	0.0001	0.0031	-0.13	0.0523	0.1773	-0.1	0.1608	0.5744	-0.06	0.3693	0.6838	-0.04	0.54717	0.641873	0	0.95131	0.95628
GP11	-0.07	0.2842	0.4446	0.04	0.5523	0.7165	0.06	0.3751	0.7307	-0.02	0.8096	0.9775	-0.03	0.61529	0.69505	-0.08	0.20148	0.361479
GP12	0.11	0.1201	0.3382	0.04	0.5873	0.7165	0.22	0.0021	0.1226	0.1	0.1363	0.5196	-0.22	0.00118	0.003428	-0.31	0.00001	0.000122
GP13	-0.03	0.6097	0.6932	0.08	0.2367	0.4011	-0.06	0.4177	0.7307	-0.06	0.3543	0.6838	-0.05	0.48829	0.588794	0.02	0.81375	0.886406
GP14	0.16	0.0204	0.1039	0.14	0.035	0.1641	0.07	0.3500	0.7307	0.02	0.8228	0.9775	-0.08	0.26735	0.388294	-0.19	0.00406	0.014568
GP15	0.12	0.0715	0.2423	0.12	0.0632	0.1926	-0.06	0.4151	0.7307	-0.01	0.8333	0.9775	-0.11	0.09717	0.155983	-0.25	0.00013	0.000721
GP16	-0.3	0.0001	0.0031	-0.13	0.0571	0.1832	-0.12	0.0956	0.5476	-0.08	0.2114	0.5459	-0.07	0.29409	0.417197	-0.09	0.1615	0.31779
GP17	0.11	0.1009	0.3239	0	0.9864	0.9864	0.09	0.1802	0.5744	0.04	0.5413	0.8255	-0.01	0.89926	0.929743	-0.11	0.10312	0.209677
GP18	0.06	0.3832	0.5436	0	0.9531	0.985	0.1	0.1476	0.5744	0.09	0.1959	0.5459	-0.44	0.00001	6.78E-05	-0.29	0.00001	0.000122
GP19	0.09	0.1852	0.353	-0.06	0.355	0.5414	-0.02	0.7965	0.9248	-0.01	0.9055	0.9932	0.33	0.00001	6.78E-05	0.31	0.00001	0.000122
GP20	0.05	0.4823	0.6226	0	0.9689	0.985	-0.06	0.3694	0.7307	0.04	0.5277	0.8253	0.15	0.03154	0.066343	0.13	0.05636	0.127332
GP21	0.02	0.7883	0.8436	0.06	0.3476	0.5414	-0.17	0.0127	0.2582	-0.07	0.3254	0.6769	-0.07	0.3347	0.462082	-0.14	0.03232	0.085718
GP22	-0.04	0.5191	0.6333	-0.09	0.1821	0.3267	-0.13	0.0634	0.5162	-0.03	0.6064	0.8281	0.13	0.06036	0.108293	0.06	0.38805	0.55049
GP23	0.24	0.0003	0.0055	0.1	0.135	0.2941	0.01	0.8494	0.9248	-0.05	0.5026	0.8187	-0.06	0.36983	0.479992	-0.05	0.43534	0.590128
GP24	0.08	0.2217	0.3977	-0.08	0.2527	0.4166	0.15	0.0378	0.4616	0.1	0.1529	0.5283	0.06	0.34088	0.462082	0.02	0.76001	0.84292
GP25	0.1	0.1597	0.3411	0.11	0.1031	0.2566	0.2	0.0040	0.1226	0.07	0.3015	0.6568	-0.17	0.01137	0.027743	-0.13	0.04998	0.117542
GP26	0.12	0.0677	0.2423	-0.1	0.1465	0.3047	0.14	0.0457	0.4646	0.08	0.2151	0.5459	0.35	0.00001	6.78E-05	0.21	0.00126	0.00549
GP27	0.02	0.745	0.8115	0.11	0.1097	0.2574	0.01	0.8470	0.9248	0	0.9919	0.9932	-0.13	0.06408	0.111682	-0.06	0.34702	0.543275
GP28	0.07	0.2743	0.4404	-0.13	0.0459	0.1773	0.13	0.0677	0.5162	0.13	0.0465	0.5083	0	0.98094	0.98094	0	0.95628	0.95628
GP29	0.02	0.8189	0.8612	-0.09	0.1729	0.3195	0.01	0.8485	0.9248	0.03	0.6381	0.8281	-0.27	0.00005	0.000218	-0.15	0.02631	0.073394
GP30	0.05	0.477	0.6226	-0.18	0.0056	0.0854	0.08	0.2806	0.6847	0.11	0.0907	0.5083	0.22	0.00085	0.002729	0.12	0.06975	0.151955
GP31	0.15	0.0285	0.1244	-0.19	0.0035	0.072	0.12	0.0824	0.5476	0.15	0.0271	0.5083	0.34	0.00001	6.78E-05	0.2	0.00254	0.009684
GP32	0.08	0.2626	0.4329	-0.11	0.1052	0.2566	0.01	0.9379	0.9486	0.06	0.3963	0.7071	0.48	0.00001	6.78E-05	0.29	0.00001	0.000122
GP33	0.01	0.8973	0.9277	0.09	0.1666	0.3176	-0.01	0.8945	0.9248	-0.01	0.8737	0.987	-0.12	0.08016	0.132156	-0.06	0.36327	0.55049
GP34	0.2	0.0031	0.0234	-0.12	0.0663	0.1926	0.11	0.1077	0.5476	0.13	0.0573	0.5083	0.41	0.00001	6.78E-05	0.23	0.0004	0.002033
GP35	0.07	0.3184	0.4856	0.07	0.2855	0.4583	0.05	0.4581	0.7552	0.03	0.6351	0.8281	0.02	0.79949	0.886707	0.02	0.72364	0.836186
GP36	0.09	0.1678	0.3411	-0.04	0.5443	0.7165	0.16	0.0193	0.2936	0.09	0.2019	0.5459	0.27	0.00004	0.000203	0.06	0.40211	0.557471
GP37	0	0.9452	0.9452	-0.14	0.0302	0.1534	-0.01	0.8768	0.9248	0.06	0.4073	0.7071	0.05	0.49227	0.588794	-0.04	0.52142	0.691448
GP38	0.01	0.9228	0.9382	-0.16	0.0158	0.1077	0.01	0.8483	0.9248	0.07	0.2824	0.6381	0.25	0.00017	0.00061	0.06	0.34734	0.543275
GP39	0.03	0.6136	0.6932	0.1	0.1506	0.3047	-0.01	0.8881	0.9248	0	0.9932	0.9932	0.05	0.42218	0.525571	0.01	0.93665	0.95628

Supplementary Table 6.5 Correlations between derived plasma N-glycan traits and age, HDLC and BMI in cases and controls

	Age						HDL-c						BMI					
	controls			Cases			CONTROLS			CASES			CONTROLS			CASES		
	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
LB	-0.11	0.1110	0.3382	0.13	0.0508	0.1773	-0.09	0.1883	0.5744	-0.11	0.1083	0.5083	-0.19	0.0038	0.0100	-0.15	0.0227	0.0691
HB	0.1	0.1317	0.3382	-0.12	0.0737	0.2043	0.1	0.1757	0.5744	0.12	0.0787	0.5083	0.22	0.0010	0.0031	0.13	0.0501	0.1175
S0	-0.1	0.1467	0.3411	0.08	0.2188	0.3814	-0.02	0.7945	0.9248	-0.08	0.2342	0.5495	-0.12	0.0763	0.1292	-0.06	0.3873	0.5505
S1	-0.05	0.5001	0.6226	0.06	0.3943	0.5726	0.03	0.6978	0.9248	-0.03	0.6289	0.8281	-0.13	0.0484	0.0934	-0.26	0.0001	0.0004
S2	0.1	0.1525	0.3411	-0.04	0.5477	0.7165	-0.04	0.5801	0.8631	0.05	0.4173	0.7071	0.13	0.0490	0.0934	0.12	0.0755	0.1589
S3	0.1	0.1246	0.3382	-0.12	0.0792	0.2102	0.08	0.2803	0.6847	0.13	0.0619	0.5083	0.2	0.0026	0.0073	0.14	0.0403	0.1025
S4	0.05	0.4709	0.6226	-0.09	0.1549	0.3047	0.03	0.7072	0.9248	0.04	0.5100	0.8187	0.16	0.0171	0.0386	0.02	0.7331	0.8362
G0	0.19	0.0043	0.0289	0.17	0.0101	0.0882	0.06	0.4119	0.7307	0	0.9742	0.9932	-0.15	0.0242	0.0527	-0.08	0.2535	0.4331
G1	-0.15	0.0257	0.1207	0.04	0.5655	0.7165	-0.02	0.7517	0.9248	-0.1	0.1559	0.5283	-0.06	0.3524	0.4672	0	0.9561	0.9563
G2	-0.09	0.1658	0.3411	-0.02	0.7983	0.8854	-0.08	0.2686	0.6847	-0.07	0.3329	0.6769	0	0.9434	0.9591	-0.08	0.2556	0.4331
G3	0.09	0.1768	0.3480	-0.16	0.0177	0.1077	0.1	0.1379	0.5744	0.13	0.0630	0.5083	0.26	0.0001	0.0005	0.15	0.0265	0.0734
G4	0.04	0.5904	0.6926	0.05	0.4669	0.6472	0.02	0.8036	0.9248	0.02	0.7745	0.9642	0.01	0.8460	0.9054	-0.03	0.6768	0.8362
FUC_A	0.13	0.0510	0.2075	-0.03	0.6533	0.7664	0	0.9486	0.9486	0.03	0.6155	0.8281	0.33	0.0000	0.0001	0.23	0.0006	0.0027
FUC_C	-0.12	0.0703	0.2423	0.04	0.5857	0.7165	-0.04	0.5708	0.8631	-0.09	0.1748	0.5459	-0.08	0.2501	0.3722	-0.02	0.7402	0.8362
BA	-0.1	0.1386	0.3382	0.13	0.0480	0.1773	-0.09	0.1990	0.5780	-0.11	0.1067	0.5083	-0.23	0.0007	0.0023	-0.16	0.0139	0.0472
A2	0.2	0.0028	0.0234	0.17	0.0096	0.0882	0.06	0.4317	0.7314	0	0.9752	0.9932	-0.16	0.0156	0.0366	-0.07	0.3047	0.5024
A2G	-0.21	0.0017	0.0207	-0.01	0.8959	0.9588	-0.1	0.1756	0.5744	-0.11	0.1065	0.5083	-0.08	0.2430	0.3705	-0.09	0.1895	0.3512
BAMS	-0.05	0.5001	0.6226	0.06	0.3943	0.5726	0.03	0.6978	0.9248	-0.03	0.6289	0.8281	-0.13	0.0484	0.0934	-0.26	0.0001	0.0004
BADS	0.08	0.2607	0.4329	-0.01	0.8560	0.9325	-0.07	0.3533	0.7307	0.03	0.6312	0.8281	0.08	0.2363	0.3696	0.09	0.1900	0.3512
TRIA	0.09	0.1932	0.3571	-0.16	0.0165	0.1077	0.1	0.1438	0.5744	0.12	0.0785	0.5083	0.27	0.0000	0.0002	0.16	0.0173	0.0554
TA	0.04	0.5513	0.6594	0.05	0.4470	0.6342	0.02	0.7850	0.9248	0.02	0.7528	0.9566	0.01	0.8307	0.9049	-0.02	0.7260	0.8362
DG9ind	0.08	0.2470	0.4305	0.02	0.7310	0.8413	-0.02	0.7761	0.9248	0	0.9578	0.9932	0.44	0.0000	0.0001	0.28	0.0000	0.0001

Appendix III

Chapter Eight Supplementary Data

Supplementary Table 8.1 Correlations between derived plasma N-glycan traits and TC, TG, HDL-c and LDL-c at baseline and follow up

	TC						TG						HDL-c						LDLC		
	Baseline			Follow up			Baseline			Follow up			Baseline			Follow up			Baseline		
	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
LB	-0.1	0.201	0.589	-0.17	0.029	0.092	-0.15	0.072	0.290	-0.22	0.007	0.054	-0.12	0.135	0.535	-0.11	0.154	0.598	-0.08	0.328	0.834
HB	0.11	0.162	0.589	0.18	0.029	0.092	0.16	**0.046**	0.290	0.22	**0.006**	0.054	0.12	0.131	0.535	0.12	0.148	0.598	0.09	0.285	0.834
S0	-0.1	0.241	0.603	-0.17	0.030	0.092	-0.07	0.399	0.290	-0.15	0.059	0.187	-0.15	0.061	0.535	-0.07	0.394	0.780	-0.07	0.366	0.834
S1	0.05	0.533	0.799	-0.02	0.827	0.871	-0.03	0.709	0.968	-0.05	0.568	0.775	-0.04	0.664	0.972	0.04	0.640	0.891	0.06	0.487	0.834
S2	0.09	0.288	0.649	0.16	0.042	0.114	0.1	0.241	0.325	0.13	0.101	0.247	0.12	0.130	0.535	0.01	0.935	0.956	0.06	0.448	0.834
S3	0.09	0.292	0.649	0.14	0.073	0.140	0.14	0.084	0.348	0.21	0.008	0.054	0.13	0.104	0.535	0.1	0.217	0.598	0.06	0.443	0.834
S4	0.04	0.603	0.830	0.12	0.131	0.207	0.14	0.076	0.414	0.07	0.378	0.597	0.02	0.790	0.993	0.1	0.218	0.598	0.02	0.844	0.890
G0	-0.03	0.732	0.833	-0.08	0.319	0.415	0.01	0.905	0.676	-0.01	0.928	0.955	-0.01	0.910	0.993	-0.03	0.684	0.891	-0.04	0.603	0.834
G1	-0.11	0.175	0.589	-0.16	0.051	0.133	-0.11	0.199	0.348	-0.15	0.054	0.187	-0.19	0.017	0.507	-0.12	0.126	0.598	-0.07	0.413	0.834
G2	0.05	0.570	0.815	0.01	0.888	0.888	-0.04	0.648	0.968	-0.03	0.685	0.874	-0.02	0.829	0.993	-0.05	0.516	0.836	0.06	0.449	0.834
G3	0.15	0.063	0.551	0.21	0.009	0.069	0.22	**0.007**	0.275	0.21	**0.009**	0.055	0.12	0.144	0.535	0.11	0.162	0.598	0.11	0.177	0.834
G4	-0.03	0.676	0.833	-0.07	0.397	0.497	-0.04	0.621	0.725	-0.01	0.863	0.955	0.00	0.986	0.993	0.01	0.906	0.956	-0.02	0.806	0.885
FUC_A	0.01	0.867	0.897	0.06	0.461	0.532	0.18	**0.029**	0.725	0.13	0.118	0.273	0.07	0.376	0.704	0.06	0.442	0.780	-0.01	0.871	0.901
FUC_C	-0.1	0.225	0.589	-0.14	0.077	0.144	-0.07	0.417	0.348	-0.14	0.079	0.225	-0.14	0.082	0.535	-0.1	0.202	0.598	-0.07	0.378	0.834
BA	-0.12	0.144	0.589	-0.2	0.012	0.069	-0.16	**0.045**	0.275	-0.23	**0.005**	0.054	-0.12	0.147	0.535	-0.12	0.152	0.598	-0.09	0.261	0.834
A2	-0.04	0.609	0.830	-0.09	0.275	0.384	0.00	0.958	0.676	-0.01	0.933	0.955	-0.01	0.883	0.993	-0.03	0.670	0.891	-0.05	0.504	0.834
A2G	-0.02	0.791	0.833	-0.02	0.827	0.871	-0.03	0.709	0.968	-0.05	0.568	0.775	-0.04	0.664	0.972	0.04	0.640	0.891	0.06	0.487	0.834
BAMS	0.05	0.533	0.799	0.12	0.153	0.235	0.06	0.495	0.454	0.1	0.212	0.397	0.11	0.180	0.535	-0.04	0.657	0.891	0.04	0.643	0.839
BADS	0.06	0.496	0.792	0.22	0.005	0.062	0.22	**0.007**	0.275	0.21	**0.008**	0.054	0.12	0.154	0.535	0.11	0.179	0.598	0.12	0.154	0.834
TRIA	0.16	0.052	0.551	-0.07	0.375	0.479	-0.05	0.579	0.725	-0.01	0.893	0.955	0.00	0.986	0.993	0.01	0.892	0.956	-0.02	0.800	0.885
TA	-0.03	0.671	0.833	-0.01	0.875	0.888	0.17	0.033	0.968	0.04	0.591	0.788	0.06	0.500	0.810	-0.09	0.275	0.660	-0.03	0.683	0.845

Supplementary Table 8.2 Correlations between derived plasma N-glycan traits and Age, BMI, WHtR and FPG at baseline and follow-up

	Age						BMI						WHtR						FPG					
	Baseline			Follow up			Baseline			Follow up			BASELINE			FOLLOW UP			Baseline			Follow up		
	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
LB	0.15	0.054	0.235	0.15	0.061	0.216	-0.22	0.007	**0.025**	-0.26	**0.001**	**0.004**	-0.21	0.010	**0.031**	-0.24	0.003	**0.010**	-0.18	0.024	0.114	-0.15	0.065	0.163
HB	-0.13	0.104	0.291	-0.15	0.055	0.216	0.18	0.026	0.068	0.25	**0.002**	**0.005**	0.20	0.013	**0.036**	0.24	0.003	**0.010**	0.21	0.011	0.114	0.17	0.033	0.124
S0	0.09	0.258	0.508	0.12	0.127	0.375	-0.1	0.230	0.369	-0.15	0.072	0.128	-0.05	0.496	0.709	-0.13	0.105	0.211	-0.14	0.074	0.221	-0.16	0.051	0.161
S1	0.13	0.108	0.291	0.07	0.384	0.563	-0.32	0.000	**0.001**	-0.29	0.000	**0.001**	-0.31	0.000	**0.001**	-0.23	0.005	**0.013**	-0.01	0.947	0.974	-0.02	0.801	0.889
S2	-0.06	0.460	0.668	-0.11	0.188	0.435	0.14	0.072	0.142	0.17	0.036	0.070	0.09	0.241	0.438	0.12	0.126	0.229	0.08	0.306	0.560	0.15	0.059	0.161
S3	-0.13	0.099	0.291	-0.18	**0.024**	0.146	0.18	0.024	0.066	0.27	0.001	**0.003**	0.19	0.019	**0.046**	0.24	0.002	**0.010**	0.20	0.011	0.114	0.17	0.033	0.124
S4	-0.09	0.253	0.508	0.02	0.832	0.876	-0.02	0.819	0.847	0.03	0.689	0.778	0.04	0.599	0.756	0.05	0.510	0.666	0.05	0.539	0.703	-0.02	0.842	0.893
G0	0.18	**0.024**	0.189	0.22	**0.006**	0.090	-0.05	0.552	0.701	-0.08	0.326	0.442	0.04	0.589	0.756	0.00	0.994	0.994	-0.06	0.459	0.672	-0.06	0.443	0.605
G1	0.07	0.404	0.632	0.08	0.297	0.558	-0.08	0.334	0.484	-0.10	0.213	0.317	-0.03	0.696	0.803	-0.11	0.158	0.256	-0.15	0.062	0.208	-0.19	0.016	0.104
G2	-0.05	0.508	0.685	-0.08	0.341	0.563	-0.12	0.134	0.240	-0.10	0.235	0.341	-0.19	0.015	**0.039**	-0.14	0.092	0.190	-0.01	0.928	0.974	0.03	0.754	0.885
G3	-0.18	**0.028**	0.189	-0.18	**0.022**	0.146	0.2	0.012	**0.037**	0.26	0.001	**0.003**	0.22	0.005	**0.021**	0.23	0.004	**0.013**	0.23	0.005	0.100	0.24	0.003	**0.033**
G4	0.03	0.726	0.849	0.08	0.343	0.563	-0.04	0.661	0.776	0.00	0.989	0.995	-0.02	0.810	0.868	0.04	0.656	0.757	-0.02	0.831	0.959	-0.13	0.110	0.219
FUC_A	-0.05	0.556	0.696	-0.04	0.581	0.717	0.2	0.013	**0.040**	0.31	0.000	**0.001**	0.27	0.001	**0.005**	0.29	0.000	**0.001**	0.12	0.129	0.324	0.14	0.085	0.191
FUC_C	0.05	0.497	0.685	0.07	0.376	0.563	-0.08	0.320	0.476	-0.16	0.047	0.090	-0.04	0.662	0.794	-0.16	0.051	0.117	-0.08	0.304	0.560	-0.09	0.284	0.405
BA	0.16	0.052	0.235	0.15	0.058	0.216	-0.22	0.005	**0.021**	-0.26	0.001	**0.003**	-0.21	0.010	**0.031**	-0.25	0.002	**0.008**	-0.19	0.018	0.114	-0.15	0.062	0.161
A2	0.18	**0.022**	0.189	0.22	**0.005**	0.090	-0.04	0.633	0.757	-0.07	0.362	0.479	0.05	0.511	0.712	0.00	0.972	0.994	-0.06	0.444	0.672	-0.06	0.485	0.637
A2G	-0.01	0.909	0.969	-0.03	0.667	0.770	-0.17	0.029	0.074	-0.17	0.032	0.065	-0.24	0.002	**0.011**	-0.24	0.003	**0.010**	-0.11	0.161	0.387	-0.12	0.152	0.260
BAMS	0.13	0.108	0.291	0.07	0.384	0.563	-0.32	0.000	**0.001**	-0.29	0.000	**0.001**	-0.31	0.000	**0.001**	-0.23	0.005	**0.013**	-0.01	0.947	0.974	-0.02	0.801	0.889
BADS	-0.03	0.730	0.849	-0.06	0.430	0.575	0.09	0.286	0.436	0.12	0.125	0.207	0.04	0.605	0.756	0.08	0.311	0.434	0.04	0.609	0.746	0.11	0.168	0.263
TRIA	-0.18	**0.027**	0.189	-0.19	**0.018**	0.146	0.21	0.009	**0.032**	0.27	0.001	**0.003**	0.22	0.005	**0.021**	0.23	0.004	**0.013**	0.23	0.004	0.100	0.23	0.003	**0.033**
TA	0.03	0.698	0.849	0.08	0.341	0.563	-0.03	0.701	0.792	0.00	0.961	0.995	-0.02	0.849	0.894	0.04	0.598	0.732	-0.01	0.857	0.960	-0.13	0.105	0.218

Supplementary Table 8.3 Correlations between derived plasma N-glycan traits and HbA1c, DBP and SBP at baseline and follow-up

	HbA1c						DBP						SBP					
	BASELINE			FOLLOW UP			Baseline			Follow up			Baseline			Follow up		
HbA1c	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q	rs	p	q
LB	-0.24	0.003	**0.010**	-0.05	0.529	0.873	-0.03	0.741	0.780	-0.03	0.741	0.873	0.03	0.695	0.886	0.00	0.954	0.961
HB	0.27	0.001	**0.005**	0.08	0.294	0.826	0.05	0.551	0.740	0.05	0.551	0.768	-0.02	0.820	0.886	0.00	0.961	0.961
S0	-0.24	0.002	**0.010**	-0.04	0.599	0.873	-0.06	0.493	0.716	-0.06	0.493	0.768	-0.07	0.378	0.846	0.02	0.781	0.927
S1	0.02	0.815	0.843	-0.04	0.614	0.873	-0.16	0.041	0.522	-0.16	0.041	0.599	-0.02	0.793	0.886	-0.08	0.302	0.927
S2	0.21	0.007	**0.023**	0.06	0.457	0.873	0.12	0.137	0.561	0.12	0.137	0.627	0.11	0.164	0.697	-0.03	0.710	0.927
S3	0.27	0.001	**0.005**	0.08	0.299	0.826	0.07	0.415	0.649	0.07	0.415	0.760	0.03	0.719	0.886	-0.01	0.902	0.960
S4	0.09	0.293	0.399	0.00	0.989	0.989	-0.1	0.211	0.561	-0.1	0.211	0.627	-0.11	0.178	0.697	0.07	0.408	0.927
G0	-0.17	0.036	0.079	0.00	0.955	0.989	0.04	0.598	0.740	0.04	0.598	0.768	0.05	0.546	0.886	0.13	0.118	0.927
G1	-0.26	0.001	**0.006**	-0.05	0.543	0.873	-0.12	0.151	0.561	-0.12	0.151	0.627	-0.11	0.165	0.697	0.02	0.812	0.927
G2	0.09	0.277	0.387	0.00	0.980	0.989	0.00	0.987	0.987	0.00	0.987	0.987	0.05	0.505	0.886	-0.09	0.262	0.927
G3	0.30	0.000	0.002	0.12	0.128	0.583	0.10	0.236	0.561	0.10	0.236	0.627	-0.03	0.698	0.886	-0.03	0.740	0.927
G4	-0.01	0.949	0.965	-0.08	0.322	0.841	-0.15	0.063	0.522	-0.15	0.063	0.599	-0.02	0.810	0.886	0.08	0.349	0.927
FUC_A	0.21	0.009	**0.028**	0.09	0.255	0.826	0.04	0.604	0.740	0.04	0.604	0.768	0.08	0.350	0.846	0.02	0.849	0.927
FUC_C	-0.19	0.017	**0.047**	0.00	0.971	0.989	-0.03	0.688	0.763	-0.03	0.688	0.839	-0.03	0.665	0.886	0.02	0.832	0.927
BA	-0.24	0.002	**0.010**	-0.06	0.462	0.873	-0.03	0.674	0.763	-0.03	0.674	0.838	0.03	0.708	0.886	-0.02	0.810	0.927
A2	-0.17	0.039	0.085	-0.01	0.899	0.989	0.04	0.604	0.740	0.04	0.604	0.768	0.05	0.536	0.886	0.12	0.124	0.927
A2G	-0.10	0.214	0.321	-0.07	0.358	0.873	-0.08	0.321	0.561	-0.08	0.321	0.726	-0.02	0.825	0.886	-0.11	0.180	0.927
BAMS	0.02	0.815	0.843	-0.04	0.614	0.873	-0.16	0.041	0.522	-0.16	0.041	0.599	-0.02	0.793	0.886	-0.08	0.302	0.927
BADS	0.16	0.052	0.108	0.04	0.580	0.873	0.09	0.242	0.561	0.09	0.242	0.627	0.12	0.133	0.697	-0.03	0.707	0.927
TRIA	0.30	0.000	**0.002**	0.12	0.132	0.583	0.10	0.219	0.561	0.1	0.219	0.627	-0.03	0.722	0.886	-0.02	0.785	0.927
TA	0.00	0.971	0.971	-0.07	0.369	0.873	-0.15	0.069	0.522	-0.15	0.069	0.599	-0.02	0.828	0.886	0.07	0.361	0.927

Appendix IV

Research Questionnaire for Type II Diabetes Mellitus

Patient ID ……Cat file No…… Date……

Please answer every question. If you are uncertain about how to answer a question then do the best you can, but please do not leave a question blank.

Demographics

1. Age.....
2. Gender

[ ] Male
[ ] Female

3. Height…….. cm and weight (shoeless)…….. Kg?
4. Waist circumference……cm and hip…… cm?
5. Resting blood pressure?
   a. Systolic……....
   b. Diastolic ……..
6. What is your marital status?

[ ] Married
[ ] Never married
[ ] Divorced
[ ] Separated

7. What is the highest level of education you have completed?

[ ] Tertiary
[ ] Senior high school
[ ] Junior high school
[ ] Lower primary
[ ] No formal education

8. Which of the following best describes your occupation?

[ ] Employed (Including self-employed)
[ ] Retired
[ ] Keeping house
[ ] Student
[ ] other (please, specify type of job)……..

9. What is the level of your daily physical activity

[ ] Primarily sedentary (sitting down)
[ ] Sedentary with frequent activity (sitting mostly but getting up several times an hour)
[ ] Primarily physical (e.g. manual handling, mostly walking)
[ ] Physical with high intensity activity (e.g. cycling, heavy work)

10. During the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time? _____ days per week No walking in leisure time

11. How much time did you usually spend on one of those days walking in your leisure time? _____ hours per day _____ minutes per day

Suboptimal Health Status

Domain 1: Fatigue

12. **In the past 3 months**, how often were you exhausted without greatly increasing your physical activity?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

13. **In the past 3 months**, how often did you have fatigue which could not be substantially alleviated by rest?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

14. **In the past 3 months**, how often were you lethargic in your daily life?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

15. **In the past 3 months**, how often did you suffer from headaches?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

16. **In the past 3 months,** how often did you suffer from dizziness?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

17. **In the past 3 months**, how often did your eyes ache or feel tired?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

18. **In the past 3 months**, how often did your muscles or joints feel stiff?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

19. **In the past 3 months**, how often did you have pain in your shoulders / neck / back?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

20. **In the past 3 months**, how often did you have a heavy feeling in your legs when walking?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

Domain 2: Cardiovascular System

21. **In the past 3 months**, how often did you feel out of breath while resting?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

22. **In the past 3 months**, how often did you suffer from chest congestion?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

23. **In the past 3 months**, how often were you bothered by heart palpitations?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

Domain 3: Digestive System

24. **In the past 3 months**, how often was your appetite poor?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

25. **In the past 3 months**, how often did you suffer from heartburn?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

26. **In the past 3 months**, how often did you suffer from nausea?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

Domain 4: Immune System

27. **In the past 3 months**, how often did you have difficulty tolerating hot and cold temperatures?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

28. **In the past 3 months**, how often did you catch a cold?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

29. **In the past 3 months**, how often did you suffer from a sore throat?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

Domain 5: Mental Health

30. **In the past 3 months**, how often did you have difficulty falling asleep?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

31. **In the past 3 months**, how often were you troubled by waking up during the night?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

32. **In the past 3 months**, how often did you have trouble with your short-term memory?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

33. **In the past 3 months**, how often did you have difficulty responding to situations quickly or making decisions?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

34. **In the past 3 months**, how often did you have difficulty concentrating?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

35. **In the past 3 months**, how often were you distracted for no reason?

[1] Always
[2] Very often
[3] Often
[4] Occasionally
[5] Never or almost never

36. **In the past 3 months**, how often did you feel nervous or jittery?

[1] Always
[2] Very often
[3] Often
[4] Occasionally

[5] Never or almost never

Please, if you are not a type II diabetes patient, ***go to next section Q42***

Medical History and Type II Diabetes Complications

37. Has either of your parents or any of your parents, brothers or sisters been diagnosed with diabetes (Type 1 or 2), hypertension, metabolic syndrome or dyslipidaemia?

[ ] Yes
[ ] No

38. Within the past 12 months, has your doctor told you that you have high blood pressure?

[ ] Yes
[ ] No

39. How old were you when you were first told that you had hypertension or high blood pressure?.....

40. For how long have you been diagnosed of diabetes?

[ ] less than a year
[ ] 1-5 years
[ ] 6-10 years
[ ] 11-15 years
[ ] 20-25 years
[ ] 25-30 years
[ ] other specify…….

41. Are you currently or have you ever suffered from any of these diseases?.....
Please tick

[ ] stroke
[ ] neuropathy
[ ] glaucoma
[ ] erectile dysfunction
[ ] myocardial infarction
[ ] diabetic coma
[ ] coronary artery disease
[ ] retinopathy
[ ] peripheral ulcers
[ ] others e.g. cancers, arthritis, infectious diseases etc……..

Environmental, Dietary and Lifestyle Factors

42. Have you ever smoked? If no, **go to next section Q 45**

[ ] Yes
[ ] No

43. Do you currently smoke any tobacco products such as cigarettes, cigars, or pipes?

[ ] Yes
[ ] No

44. For how many years have you been smoking daily?.....

45. Have you ever consumed a drink that contains alcohol (such as beer, wine, etc.)?

[ ] Yes
[ ] No, *then* ***skip to next section*** **Q47**

46. In your life, how often do you drink alcohol?

[ ] Never or almost never
[ ] Occasionally
[ ] Often
[ ] Very often
[ ] Always

Food Preference Questions

47. How often, in the past 3 months, did you eat the following?	never	Less than 1 time per week	1-6 times per week	1-3 times per day	4 or more times per day
Dairy (cheese, milk, yogurt etc.)					
Poultry (chicken, turkey, duck etc.)					
Fish and Sea food (tilapia, tuna, shrimp, crab, etc.)					
Pork					
Beef					
Eggs					
Other meat (lamb, bush meat, venison, etc.)					
Fruits (apples, bananas, pawpaw, oranges, etc.)					
Sweets and soft drinks					
Vegetables (carrots, okro, green leafy vegetables mushrooms, potatoes, cabbage, tomatoes, Cucumber, potatoes, mushrooms, garlic, garden eggs, lettuce, cucumber, etc.)					
Cereals and cereal products (millet, maize, sorghum, oats, bread, wheat, porridge, rice)					
Fatty foods [(margarine, butter, pan fry foods (eggs, rice, potatoes meat, poultry, lamb, pork etc.)]					
Starchy foods (cassava, plantain, cocoyam)					

Skip the following questions if you are not suffering from Type II diabetes mellitus

Medications

48. How do you currently manage or control your diabetes?

[ ] Diet and/or exercise only
[ ] Oral medications
[ ] Insulin injection
[ ] Insulin pump
[ ] Other (*Specify*)……

49. Because of your high blood pressure/hypertension, have you ever been told to take prescribed medicine?

[ ] Yes
[ ] No

50. Have you ever been told by a doctor or other health professional that your blood cholesterol level was high?

[ ] Yes
[ ] No

51. Have you ever been told by a doctor or other health professional to take prescribed medicine to lower your blood cholesterol?

[ ] Yes
[ ] No

52. Please list below all prescription medicines and over-the counter medications you have been taking **for the past 3 months.**

Medicine	reasons for taking	Amount/frequency
……	……	……
…….....	……	……...……
…….	**..... .....**	**.....**
…… **.....**	**.....**	**.....**
……..	……..	……
……..	…….....	……

Appendix V

Other Supporting Documents

Participants Information Letter

Validation of N-glycan profiles as risk stratification biomarker for type II diabetes mellitus

Investigator: Eric Adua

This is a collaborative research between Edith Cowan University and the Komfo Anokye Teaching Hospital (KATH). This collaborative research entitled Validation of N-glycan profiles as a risk stratification biomarker for Type II Diabetes Mellitus (T2DM), will lead to the award of a PhD degree in Medical Sciences.

You are being invited to participate in this study because you have T2DM. Please read the information carefully and ask any questions you might have. You may also wish to discuss the study with a relative or friend or your doctor.

We wish to identify a sugar called N-glycan, which could be used as an indicator for T2DM onset, its progression and the associated risk factors that contributes to its development. If you choose to participate, we will first ask you to fast overnight from 9 pm to 8 am and then you will answer a simple validated questionnaire about your present health status, dietary pattern, medication history and family history of diabetes and cardiovascular diseases. Following this, we will draw 10 ml of your blood for laboratory testing. The entire process will take approximately 1 hr to complete and the potential benefit is that you will be able to know your medical status. Copies of the test results will be given you for you to take to your doctor if need be.

If you agree to participate in the study, you will be compensated with $10 for your time and trouble. Participation in this study is voluntary. You do not have to participate if you do not want to and your decision to participate or not will in no way affect your current or future care at KATH. You are also free to withdraw from the study at any time without reason or justification. All the information you provide will be confidential. Your data will be stored in a password protected hard drives and kept in special cabinets in the principal supervisor's office. All your data will be destroyed after five years. If the results of the trial are published in a medical journal, as is intended, no reader will be able to identify you.

There are no foreseeable major risks or side effects associated with your participation. You will however experience a minimal discomfort during the blood withdrawal stage. In the event that you suffer an expected or unexpected side effect or medical accident during this study, you will be offered

all full and necessary treatment by KATH. The ECU Human Research Ethics Committee (ECU HREC) and the Committee On Human Research, Publications And Ethics (CHRPE) of KATH has approved this study on the basis that the reported risk of such an event is either small or acceptable in terms of the risk you face as a result of your current illness.

Contact Information

If you have questions about this study, please contact

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CONSENT FORM

Validation of N-glycan profiles as risk stratification biomarker for type II diabetes mellitus

Investigator: Eric Adua, Edith Cowan University

I, …….. agree to participate in the above study. I have been provided with a copy of the participant information letter explaining the study which I have read and understood. I have been given the opportunity to ask questions about the study by the Investigator and any questions have been answered to my satisfaction. I am aware that if I have additional questions, I can contact the research team. I understand that I will be required to fast overnight between 9 am and 8 am, answer questionnaire and provide a 10 ml of venous blood sample for laboratory testing. I understand that I may withdraw from the study at any time without affecting any future medical treatment, or the treatment of the condition which is the subject of the study. I am aware that all research data collected will only be used for the purpose of this study and will be kept confidential and that my participation will not be disclosed without my consent.

Signed ……. **Date** ……

Signature ……. **Date** ……

of Investigator

Appendix VI

Standard Operating Procedures

N-glycan profiles as a risk stratification biomarker for type II diabetes mellitus and associated metabolic risk factors

General guidelines

- Before starting any blood sampling, strict hygienic conditions should be maintained.
- The work bench should be disinfected with 70% ethanol.
- New and sterile needles and syringes should be used on each participant and no two individuals should share needles or syringes.
- After each use, needles and syringes should be disposed off appropriately into sharps bin. Waste disposal should strictly follow the procedures stipulated by Komfo Anokye Teaching Hospital (KATH).
- A sterile environment should always be maintained during blood collection. All personnel should always wear personal protective equipments (PPEs) which include a comfortable laboratory coat, eye-wear and sterile gloves to protect themselves from contact with patient blood samples.
- It is also mandatory for laboratory staff to carry out proper hand washing procedures before and after each blood collection session prior to carrying out any other task as part of their protocol.
- Hygienic materials including laboratory coats, hand washing soaps, alcohol, sterile gloves and cotton wools must always be available before and after every venipuncture.
- Infection-free or 70% alcohol-wiped tourniquets should be used for each venipuncture to prevent possible transmission of infection between the phlebotomist and the participant.
- Before each venipuncture, a 70% alcohol swap is used to clean the arm of each participant before drawing blood.
- To avoid swapping samples and cross-contamination, blood samples must be collected into vacutainer tubes and labelled immediately before arranging on a rack.

Equipment, chemicals and consumables required

Equipment

- Centrifuge

- Freezer (-80^{o}C)
- 1 ml pipette
- Tourniquet
- Biochemical/test kits
- Permanent markers

Consumables

- Vacutainer tubes
- Cryotubes 1.8 ml
- 1 ml pipette tips
- 2 ml eppendorf tubes
- Sterile needles
- Cotton wools

Timeline for blood collection procedures

Procedure	Time
1. Collection of one patient blood sample	20 min
2. Transfer to laboratory	2 hrs
3. Leave the tube resting at room temperature	20 min
4. Centrifugation of plasma	10 min
5. Transfer of plasma	10 min
6. Freezing of plasma	5 min
Approximate total time for procedure	**3 hrs, 5 min**

Method

Collection of blood samples

- Collect blood sample from each patient using a tourniquet + butterfly needle method.
- 10 ml of whole blood should be collected into two EDTA vacutainer tubes (5 ml each) and inverted 3X to ensure uniform mixture.
- One tube will be used for routine biochemical tests including (LDL, cholesterol, triglycerides, C-reactive proteins, insulin, glucose and glycated hemoglobin).
- The other tube will be processed for genetic and N-glycan analysis.

Labelling

- Note participant/patient ID and the date on each questionnaire.
- Clearly mark the patient ID on both the vacutainer tubes and the cryotubes.

NOTE: Make sure the labelling on the questionnaire corresponds to that on the tube!!

Transfer of sample to laboratory

Place tubes in cool boxes packed with ice blocks/ freezing gels (4^{o}C) and sent to the laboratory within 4 hours its draw.

Time of arrival ……lab technician…….Date…….

NOTE: Samples must be processed on the same day upon arrival at the laboratory!!!

Centrifugation of plasma

- Leave the tube resting at the room temperature for 20-25 mins to prevent samples from being hemolysed.
- Place the tube in a centrifuge and spin.
- Ensure tube is balanced with equivalent water containing tube.
- Set centrifuge to spin for 10 mins at 1620 g or the equivalent rpm at 4^{o}C.
- Check if samples are hemolysed and note it.

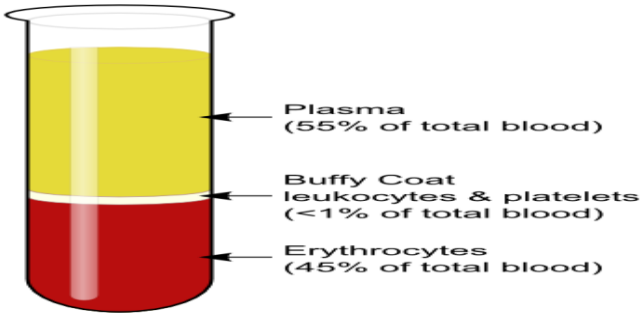

Figure 1. The components of blood

Aliquoting plasma and buffy coat

- Transfer plasma to a 1.8 ml microcryotubes tubes.
  - label as PLS + Patient ID
- Carefully aliquot buffy coat into new 1.8 ml microcryotubes.
  - Label microcryotubes as BC + Patient ID

NOTE: Make the patient ID on both microcryotubes corresponds with each other!!!

Deviations from procedure

Note any deviations from the procedure here, giving reasons and effects. Include sample details.

……
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Storage

- Processed plasma and buffy coats in microcryotubes should be in clearly labelled plastic containers.
- Place plastic containers in plastic bags.
- Store at a specific space in a -80^{o}C freezer.
- Note samples in freezer log book.
- Participant information and details of each sample should be noted in a processing notebook.
- Export data into an Excel spreadsheet or a Microsoft access database.

Sample transportation

The transport of biosamples has to be performed strictly under standardized conditions to prevent a loss of sample quality.

Sample packing

- Each tube/vial should be identified with printed labels including sample type.
- Tubes should be packed in cardboard/plastic boxes, ideally in a Styrofoam box with a coating thickness of at least 5 cm for adequate stability.
- A coated paperboard box is favored over a non-coated box.
- Avoid packing tubes in plastic bags directly.
- Paper toweling can be placed in the box to cushion the sample tubes while transporting.

- The paperboard or Styrofoam boxes must be labeled with the required hazardous material tags.
- Mark packaged boxes with information concerning sender and recipient of the biosample delivery (address, contact person).

Sample shipping

- Ship plasma and Buffy coat samples on dry ice.
- Ensure that the samples are properly packed to maintain the required temperature for the journey plus two days.
- The biosamples should be surrounded from all sides by a dry ice layer with a thickness of at least 5 cm.
- Vacuity above the dry ice layer should be filled-up with packing material or further dry ice in order to avoid a shift of the insulating bed (dry ice) during the transport.
- For reasons of dispersal, dry ice pellets (nuggets) are favored over dry ice blocks.

Shipping process

Before shipping, please inform the recipient on the following information:

- Mark contact details including sender and recipient name and phone numbers on the package.
- Shipping details (shipping company, intended shipping date, shipment packaging and temperature).
- Sample details (total number of samples, complete list of samples).
- After shipping, inform the recipient on waybill number for tracking of shipment.
- An acknowledgement will be sent to the shipper when the samples have been received and checked.
- The process is not completed till the recipient confirms the acceptance of the consignment.