Leukocyte O-GlcNAcylation: A novel diagnostic tool for the earlier detection of type 2 diabetes mellitus?

Clare Springhorn

Dissertation presented for the Degree of

MASTERS

In Physiological Sciences at Stellenbosch University

Supervisor: **Prof. M. Faadiel Essop**

December 2012

Cardio-Metabolic Research Group

Declaration

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Abstract

Context: There are serious deficiencies in the current tests and criteria available for the diagnosis of diabetes. A novel screening method for the earlier and more efficient detection of type 2 diabetes would be a significant clinical advance.

Objective: The hexosamine biosynthetic pathway (HBP) usually acts as a fuel sensor and its activation leads to *O*-GlcNAcylation of target proteins in a glucose-responsive manner. *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) are responsible for *O*-GlcNAc addition and removal, respectively. As higher HBP flux is linked to insulin resistance/type 2 diabetes, we hypothesized that increased *O*-GlcNAcylation of leukocyte proteins can detect the onset of pre- and overt diabetes.

Materials and methods: 74 participants from Bellville and Stellenbosch (Western Cape, South Africa) were recruited and characterized as normal, pre-diabetic or diabetic. Leukocytes (granulocytes and lymphocytes) isolated from study subjects were evaluated for *O*-GlcNAcylation, OGA and OGT expression by flow cytometry, immunofluorescence microscopy and Western blotting.

Results: Leukocyte *O*-GlcNAcylation increased in both pre-diabetic and diabetic individuals, with leukocyte sub-population data showing the greatest sensitivity. OGA expression and *O*-GlcNAc/OGA ratios elevated in parallel with increasing glucose concentrations. OGT expression did not significantly change for any of the study subjects investigated.

Conclusions: The initial and significant increases in leukocyte *O*-GlcNAcylation demonstrate great potential for the earlier detection of pre-diabetic and diabetic individuals. OGA expression and O-GlcNAc/OGA ratios may also have diagnostic value. Together our data show strong promise for eventual diagnostic utility and the more efficient detection of type 2 diabetes.

Uitreksel

Die konteks: Daar is ernstige tekortkominge in die huidige toetsing en kriteria vir die diagnose van diabetes. 'n Nuwe metode vir die vroeë en meer effektiewe opsporing van tipe 2 diabetes sal beduidende kliniese voordeel inhou.

Doelstelling: Onder normale omstandighede tree die heksosamienbiosintetiese pad (HBP) as energie sensor op, en die aktivering daarvan gee aanleiding tot O-GlcNAsetilering van proteïene in 'n glukose-afhanglike wyse. O-GlcNAs transferase (OGT) en O-GlcNAse (OGA) is onderskeidelik verantwoordelik vir O-GlcNAs toevoeging en verwydering. Aangesien hoër HBP fluks verband hou met insulienweerstandigheid /tipe 2 diabetes, stel ons 'n hipotese voor dat opsporing van verhoogde O-GlcNAsilasie van leukosietproteïene, die aanvang van pre-diabetes en diabetes kan voorspel.

Materiale en metodes: 74 vrywillige deelnemers van Bellville en Stellenbosch (Wes Kaap Provinsie, Suid Afrika) is gewerf en gekarakteriseer as normaal, pre-diabeties of diabeties. Leukosiete (granulosiete en limfosiete), uit bloed van deelnemers geïsoleer, is vir O-GlcNAsilasie, OGA en OGT uitdrukking deur vloeisitometrie, immunofluoressensie-mikroskopie en Western blotting, ondersoek.

Resultate: Leukosiet O-GlcNAsetilering is verhoog in beide pre-diabetiese en diabetiese individue, met leukosiet sub-populasie wat die mees sensitiewe data gelewer het. OGA uitdrukking en O-GlcNAs/OGA verhoudings in parallel verhoog tot 'n toename in glukose konsentrasies. OGT uitdrukking het nie betekenisvol verander in enige van die individue wat ondersoek is nie.

Gevolgtrekkings: Die vroeë en betekenisvolle toename in leukosiet O-GlcNAsetilering toon groot potensiaal vir die vroeë opsporing van pre-diabetiese en diabetiese individue. OGA uitdrukking en O-GlcNAs/OGA verhoudings het ook moontlik diagnostiese waarde. Ons data toon belowende resultate vir die gevolglike diagnostiese waarde en 'n meer effektiewe opsporing van tipe 2 diabetes.

Acknowledgements

I thank my supervisor, Prof Essop, for his loyalty, wisdom, motivation and guidance. Prof, thank you for all the unbelievable opportunities and for allowing me to grow and learn on my own terms. You have provided me with the foundation and skills necessary to pursue new challenges, and for this I am truly grateful.

To the best parents in the world, thank you for your unwavering support and love, there are no words to describe how much both of you mean to me and how much I appreciate everything you do for me (which is too much).

To the love of my love, thank you for your unconditional love and support, and for being the most dependable person in my life. I am indebted mostly to you!

To H and Megan, thank you for all the laughs and the Buzz coffees. I am so happy to have been able to share this experience with you. To Thomas, thank you for being a true friend and for teaching me so many valuable life lessons.

To the best research group, CMRG, thank you for being an unbelievable support system and for always being willing to help out! To my fellow colleagues, for empathy when western blots didn't work and jealousy when they did. ☺ Thank you for making the lab a fun place to work in.

To Catrina, thank you for putting a smile on my face every single morning. I will truly miss you.

Thank you to Ithemba labs and the Bellville-South team (particularly Ryan, Alain and Macharia) for help with subject recruitment, without your assistance this project would not have been possible.

Thank you to the NRF, the Harry Crossley Foundation and the Ernst & Ethel Eriksen Trust for the generous financial support.

Table of Contents

List of Figures

List of Tables

Nomenclature

β cell:	Beta cell
ADA:	American Diabetes Association
AGE:	Advanced glycation end products
AMP:	Adenosine monophosphate
AMPK:	5′-AMP-activated protein kinase
ARV:	Antiretroviral
ATP:	Adenosine triphosphate
BSA:	Bovine serum albumin
(CaMKIV):	Calcium calmodulin-dependent protein kinase I V
CoQ:	Coenzyme Q
CVD:	Cardiovascular diseases
DAG:	Diacylglycerol
EDTA:	Ethylenediaminetetraacetic acid
ETC:	Electron transport chain
FAD:	Flavin adenine dinucleotide (oxidized)
$FADH_2$:	Flavin adenine dinucleotide (reduced)
FBS:	Fetal bovine serum
FFA:	Free fatty acid
FITC:	Fluorescein isothiocyanate
FPG:	Fasting plasma glucose
FSC:	Forward angle light scatter

G-6-P:	Glucose-6-phosphate
G6PD:	Glucose-6-phosphate dehydrogenase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GFAT:	Glutamine:fructose-6-phosphate aminotransferase
GlcN-6-P:	Glucosamine-6-phosphate
GLUT4:	Glucose transporter 4
GSH:	Glutathione (reduced)
GSPx:	Glutathione peroxidase
GSSG:	Glutathione (oxidized)
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HAT:	Histone acetyl transferase
Hb:	Hemoglobin
HbA1c:	Glycated hemoglobin
HBP:	Hexosamine biosynthetic pathway
HGC:	High glucose control
HGHI:	High glucose high insulin
HGLI:	High glucose low insulin
HGMI:	High glucose medium insulin
HGPC:	High glucose positive control
HOMA:	Homeostasis model assessment
IDF:	International Diabetes Federation
IFG:	Impaired fasting glucose
IGT:	Impaired glucose tolerance
IRS-1:	Insulin receptor substrate
LGC:	Low glucose control
LGHI:	Low glucose high insulin

LGLI: Low glucose low insulin

LGMI: Low glucose medium insulin

LGPC: Low glucose positive control

MGEA5: Meningioma expressed antigen 5

MnSOD: Manganese superoxide dismutase

NAD^+: Nicotinamide adenine dinucleotide (oxidized)

NADH: Nicotinamide adenine dinucleotide (reduced)

$NADP^+$: Nicotinamide adenine dinucleotide phosphatase (oxidized)

NADPH: Nicotinamide adenine dinucleotide phosphatase (reduced)

NCD: Non-communicable disease

NCOAT: Nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase

NEFA: Non-esterified fatty acids

NFK-B: Nuclear factor kappa-beta

O_2^-: Superoxide

OGA: *O*-GlcNAcase

O-GlcNAc: *O*-linked-N-acetylglucosamine

OGT*:* *O*-linked β-*N*-acetylglucosaminyl transferase

OGTT: Oral glucose tolerance test

PARP: Poly(ADP-ribose) polymerase

PBS: Phosphate buffered solution

PDH: Pyruvate dehydrogenase

PenStrep: Penicillin-Streptomycin solution

PFK: Phosphofructokinase

PtdIns(3,4,5)P3: Phosphatidylinositol 3,4,5-triphosphate

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein kinase C

PMSF: Phenylmethanesulfonyl fluoride

PPP: Pentose phosphate pathway

PTEN: Phosphatase and tensin homolog deleted on chromosome 10

PUGNAc: O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate

QUICKI: Quantitative insulin sensitivity check

RIPA: Radioimmunoprecipitation

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SERCA2a: Sarcoplasmic calcium ATPase

SGLT: Sodium-glucose co-transporter

SSC: Linear 90° light scatter

T2DM: Type 2 diabetes mellitus

TBS: Tris buffered saline

TBS-T: TBS-Tween

TCA: Tricarboxylic acid

TPR: Tetratricopeptide repeats

UCP: Uncoupling proteins

UDP: Uridine diphosphate

UDP-GlcNAc: Uridine diphosphate *N*-acetyl glucosamine

UTP: Uridine triphosphate

WBC: White blood cell

WHO: World Health Organization

Introduction

Diabetes is an escalating health crisis and global affliction. For example, someone dies approximately every 7 seconds from diabetes-associated effects [1]. The rapidly increasing incidence and prevalence of Type 2 diabetes mellitus (T2DM) has emerged as one of the most pressing medical concerns of both developed and developing countries [1, 2]. Furthermore, at a high-level summit hosted by the United Nations General Assembly in September 2011, it was proclaimed that diabetes and other non-communicable diseases (NCDs) had reached "epidemic proportions" [3]. Diabetes can lead to a wide-range of health issues, it is one of the world's largest contributors towards mortality, disability and economic expenditure, and it is also associated with the exacerbation of poverty and hunger [4-6]. The resulting burden on society, government, health care systems and the economy is devastating and it is therefore imperative to address such complications with urgency.

1.1 The global burden

The global diabetes healthcare expenditure amounted to an enormous $465 billion in 2011. Moreover, since diabetes results in a significant loss of economic growth and labor throughput, diabetes-induced mortality and disability rates are a global concern [1, 7]. Non-communicable diseases account for 63% of total deaths worldwide, of which the largest fraction is due to diabetes, cardiovascular diseases (CVD), cancer and chronic respiratory diseases [4]. Diabetes is responsible for ~4.6 million deaths per annum, and although more prevalent in low-income countries,

no country or community is unscathed [1, 8]. The International Diabetes Federation (IDF) estimated that ~366 million people currently suffer from diabetes (85-95% of these being type 2 diabetes), and predictions indicate that this number will surge to ~522 million by 2030 [1]. Moreover, the harsh reality of these alarming statistics is that this may be an underestimation (Figure 1.1). In support, predictions made for the year 2025 (300 million) and the year 2030 (366 million) have already been exceeded [8, 9]. This exacerbates existing concerns regarding current estimations as it is very likely that these numbers will further escalate within the next few decades (Figure 1.1).

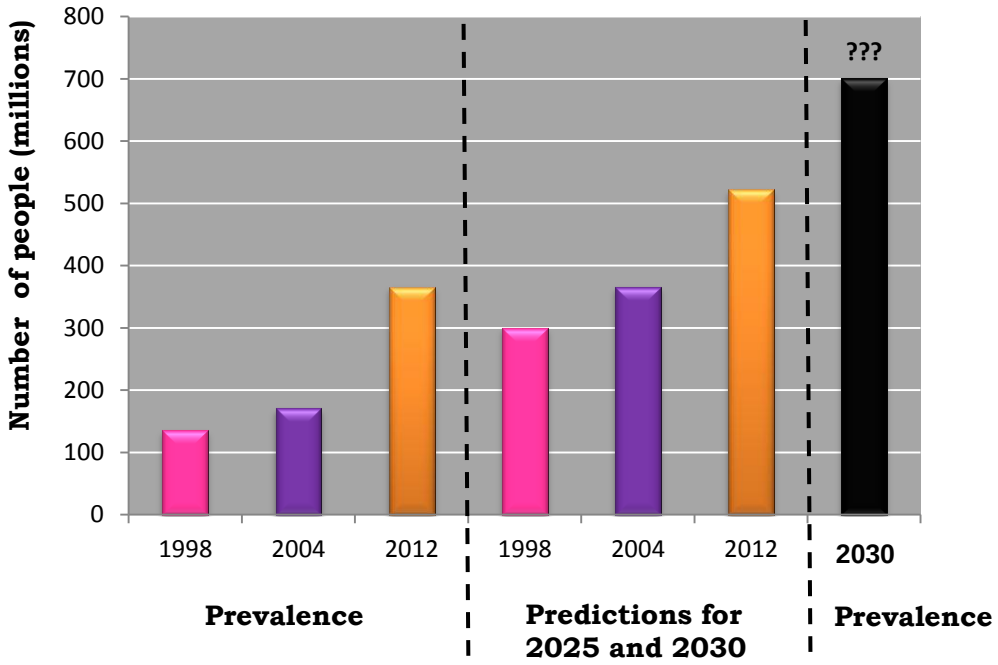


Figure 1.1 A bar graph representing the increasing prevalence of diabetes from 1998 to 2004 to 2012, and the projections made in each of these years for 2025 and 2030. As the estimations made for 2030 in 1998 and 2004 have already been surpassed, the prevalence by the year 2030 is unknown (black bar). (Generated from statistics obtained from [1, 7-9]).

Closer to home the situation is just as dire, with ~14.7 million diabetic cases reported in sub-Saharan Africa, and following a rapid upward trajectory [1, 7]. In fact, the IDF forecasts a doubling in the prevalence of diabetes in Africa between

the years 2011 and 2030 [7]. This is the alarming truth of an illness fuelled by obesity, physical inactivity, poverty, urbanization, hazardous lifestyle changes and socio-economic stressors [10, 11].

Obesity is the major contributing factor towards type 2 diabetes and glucose dysregulation, and it is predicted that by the year 2030 almost 2 billion people will be overweight [12]. Obesity is an established health challenge of the affluent nations as well as an emerging and rapidly increasing issue in non-industrialized countries [13]. Lower income countries additionally suffer from a "dual-burden" of both obesity and malnutrition [14]. With urbanization on the rise, poorer populations are limited to cheap, nutrient-deprived, carbohydrate and fat-dense foods that lead to malnourishment and/or an abundance of calories and subsequent weight gain [14, 15]. However, in India and certain Asian countries where the prevalence of obesity is relatively low, rates of type 2 diabetes are unexpectedly high [10, 16]. Here this is attributable to rapid socio-economic developments, physical inactivity and nutritional transitions resulting in greater abdominal obesity and increased insulin resistance, i.e. a "normal-weight, metabolically obese" phenotype [10].

Together, the previous discussion shows that the dynamics of type 2 diabetes are ever changing. Historically it was a condition largely prevalent in Western populations, but it now presents on a global scale [10]. Formerly known as an affliction of the rich, it is now similarly a severe problem within developing nations [15]. Furthermore, while it classically manifested in adults, type 2 diabetes is currently also an austere health challenge for younger people [17]. Thus it is emphatic that type 2 diabetes is a health issue of considerable dimension and governments worldwide are rightfully concerned since its phenotype is closely

linked to several additional debilitating illnesses, with multiple economic and societal influences [3].

1.2 Diabetes and its insidious complications

Cardiovascular diseases (CVD) remain the leading cause of deaths worldwide (Figure 1.2) [18]. Diabetes is closely associated with CVD and vascular complications are the primary cause of morbidity and mortality in diabetic sufferers [19, 20]. For example, it is the cause of mortality on more than 65% of diabetes-associated death certificates [21]. This illustrates that such associated vascular complications present a formidable challenge facing individuals with diabetes.

Patients with diabetes have an increased risk for several CVD and the progression of cardiac dysfunction may lead to coronary artery disease, hypertension, atherosclerosis, myocardial infarction and cerebrovascular disease [22-25]. Diabetic patients have an approximate 2- to 4-fold higher mortality rate compared to non-diabetics, this is with parallel vascular disease history [21]. Moreover, type 2 diabetes can elicit negative effects on cardiac structure and function in the absence of hypertension and coronary artery disease, a condition established as the diabetic cardiomyopathy [25-27].

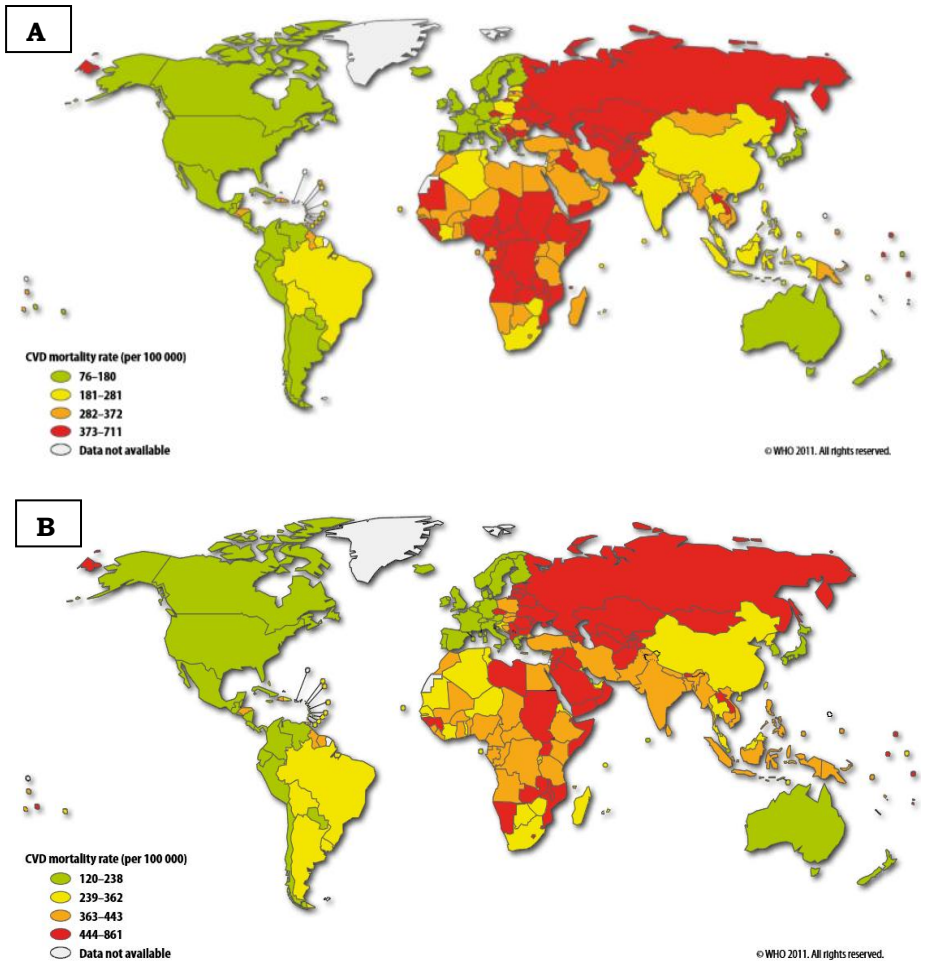


Figure 1.2 World maps presenting the global distribution of **A:** male and **B:** female deaths caused by CVD [18].

Diabetes is also associated with lower extremity amputation, retinopathy, neuropathy, several cancers, degenerative disorders and blindness [21, 23, 28-30]. Furthermore, it can exacerbate tuberculosis and HIV/AIDS, two of the world's most rampant infectious diseases [1, 31].

This compelling information therefore underscores the need for the early detection of type 2 diabetes. Undiagnosed or delayed diagnosis of diabetes often causes the progression of many of the above-mentioned diabetic complications [1]. Therefore, our premise is that the inefficient diagnosis of diabetes is a major role-player contributing to such costly and debilitating consequences.

1.3 The diagnosis of diabetes

Although current interventions are affordable and widely available, millions remain under-diagnosed and are disabled and/or die due to this illness every year [1, 32]. For example, ~25% of patients diagnosed with diabetes have already carried the disease for 4-7 years, and at the time of diagnosis present with established micro- and macrovascular complications [33]. This situation is even more alarming for the African continent where a massive 78% of diabetic individuals are undiagnosed and innumerable children die without ever being diagnosed [1].

To support the urgent need for a global response to this epidemic, we aim to emphasize the importance of increased detection of diabetes, and more importantly of the pre-diabetic condition. We will firstly focus on providing a useful, informative overview of the diagnosis of diabetes. Here we aim to provide clarity regarding the current status of diabetes diagnosis and to, more importantly, utilize this to serve as a practical platform with which to aid improved diagnosis and the delay of complications. We also aim to place the existing diagnostic criteria into prognostic perspective, thereby providing context and precision regarding current strategies that are effective. Here we will also highlight areas where advances should be targeted to best further the field of diabetes diagnosis.

1.3.1 The history of diabetes diagnosis

The earliest known evidence of diabetes is recorded in 1552 BC (on Egyptian papyrus), and is described as an illness resulting in frequent urination [34]. The timeline of its diagnosis starts as early as 600 BC when Surutus, the father of Indian medicine, diagnosed this condition as “diabetes” [34]. From around 500 BC, a physician’s positive diagnosis relied upon the level of agility and fortitude

exhibited by ants toward a specimen of urine [35-38]. Up until the eleventh century, diagnosis was confirmed by the characteristic sweet taste of a diabetic's urine. This was the task of individuals referred to as "water tasters" who had the unenviable job to drink the patient's urine. It was at this time in history when the Latin word "mellitus" (honey) was coined as part of the term "diabetes" [38-40].

During 1797 an English military doctor, John Rollo, demonstrated the presence of surplus sugar in urine and in the blood [34]. It was only in the early 1800s when researchers technologically advanced the diagnosis of diabetes through the development of the first chemical tests capable of measuring urinary sugar levels [38, 41, 42]. This was further advanced when Benedict (in 1907) formulated a novel method based on the reduction of alkaline copper solutions to detect urinary sugar [43].

The first observations of blood sugar at specific intervals after the ingestion of a test dose were made by Bang (1913) and his co-workers (discussed in [44]). Subsequently, various innovations were made between 1925 and the 1970s that allowed for accurate and easy blood glucose detection [38, 45, 46]. These included plasma "glucose brackets", dextrostix®, and the daily mean fasting plasma glucose test [38, 47, 48]. During 1965 the World Health Organization (WHO) made the first formal request for the clinical diagnosis of diabetes to be based on the oral glucose tolerance test (OGTT) (reviewed in [49]). Inception of the glycated hemoglobin (HbA1c) test occurred in 1977. However, despite the fact that HbA1c was routinely used by physicians for glucose monitoring and primarily for prognostication, it was not yet recognized as an official diagnostic tool (reviewed in [32]). Following several debates and controversies regarding HbA1c's utility and standardization (to be further discussed in section 1.3.2), it was officially endorsed as a first-line diagnostic test during 2009 (more than 30 years after its initial description)[50].

The present day criteria for the diagnosis of type 2 diabetes include FPG, HbA1c and OGTT tests [49]. This is based on the 2010 American Diabetes Association (ADA) guidelines and the 2011 WHO addendum report [49].

1.3.2 Comparisons, cut-off's and controversies

Indubitably, the field of diabetes diagnosis has made significant scientific advances since the utilization of ants' attraction towards sugar. However, there is an on-going debate and lack of consensus regarding both the preferred screening method for the detection of diabetes as well as organization-specific characterization criteria. These longstanding controversies regarding cut-off's, diagnostic yield and predictive value, along with comparisons between the various tests will be reviewed and summarized below.

1.3.2.1 OGTT and impaired glucose tolerance (IGT) versus FPG and impaired fasting glucose (IFG)

The ADA has historically more strongly supported the FPG test [48]. By contrast, the WHO favored the OGTT and endorsed it as their only diagnostic test until 1980, and their principal test until 2011 [49, 51]. For the most part of the 1900s, increased mortality rates due to microvascular complications placed specific emphasis on the earlier detection of diabetes (reviewed in [52]). Due to the FPG test considered unable to detect glycemic dysregulation early enough, the OGTT was considered the best available screening test [48].

During 1997 the ADA proposed that the classification of diabetes be made primarily through FPG and that the diagnostic threshold be lowered from 7.8 to 7.0 mmol/L, the cut-off value recommended by WHO at that time (Table 1.1) [53]. The

IFG category was intended to be analogous with IGT (2-hour blood glucose during an OGTT in range 7.8-11.1 mmol/L) (Table 1.1), and to similarly identify patients at risk of developing hyperglycemia-induced complications and/or diabetes [54].

1985 WHO guidelines	
OGTT: Normal: <7.8 mmol/L IGT: 7.8-11.0 mmol/L Diabetic: ≥11.1 mmol/L	**FPG:** Normal: NA IFG: NA Diabetic: ≥7.8 mmol/L
1997 ADA criteria	
OGTT: Normal: NA IGT: NA Diabetic: NA	**FPG:** Normal: <6.1 mmol/L IFG: 6.1-6.9 mmol/L Diabetic: ≥7.0 mmol/L
1999 WHO guidelines	
OGTT: Normal: <7.8 mmol/L IGT: 7.8-11.0 mmol/L Diabetic: ≥11.1 mmol/L	**FPG:** Normal: <6.1 mmol/L IFG: 6.1-6.9 mmol/L Diabetic: ≥7.0 mmol/L
2003 ADA criteria	
OGTT: Normal: <7.8 mmol/L IGT: 7.8-11.0 mmol/L Diabetic: ≥11.1 mmol/L	**FPG:** Normal: <5.6 mmol/L IFG: 5.6-6.9 mmol/L Diabetic: ≥7.0 mmol/L

Table 1.1 Amendments made by the WHO and ADA amendments to diagnostic criteria for the period 1985-2003 [53-56].

However, studies confirmed discordance between the new FPG categories recommended by ADA and the longstanding OGTT criteria accredited by the WHO [53]. For example, in the DECODE (Diabetes epidemiology: collaborative analysis of diagnostic criteria in Europe) study, only 40% of individuals with newly diagnosed diabetes met the FPG threshold values, 31% fulfilled only OGTT criteria, and only 28% satisfied both sets of diagnostic criteria [57]. It was soon observed that IFG and IGT could not be used interchangeably, and studies showed that FPG and OGTT each identified a different subset of diabetic individuals [56].

In 1999 the WHO adjusted their FPG cut-off value to match that of the ADA (Table 1.1). However, they still strongly encouraged use of the OGTT [54]. Although patients diagnosed exclusively by means of OGTT displayed worse prognostic outcomes regarding retinopathy and mortality, diagnoses made solely with FPG would fail to detect ~30% of diabetic individuals [56]. In keeping with the constant tussle between the ADA and WHO, the ADA revised their criteria in 2003 to include the use of the OGTT test in their diagnostic guidelines. Moreover, the IFG category was lowered to 5.6-6.9 mmol/L in an attempt to more easily identify those individuals at high risk for developing diabetes (Table 1.1) [55].

Taken together, the above discussion emphasizes the complexities of defining diabetes and the need for recurrent modifications with the availability of new and relevant information. At this juncture we cannot accurately validate which test (FPG vs. OGTT) is more efficient at diagnosing diabetes, as each test has its merits. It seems as if IGT and IFG, commonly referred to as "categories of increased risk for diabetes" or "pre-diabetes" reflect different scopes of the glycemic response, i.e. not essentially differing regarding specificity, sensitivity or predictive significance [49, 54]. For that reason it is not surprising that the combination of FPG and OGTT undoubtedly provides greater value than either test is capable of providing alone [54]. However, in practice this is not always feasible.

1.3.2.2 The official authorization of HbA1c

The ADA officially endorsed the use of HbA1c for the diagnosis of diabetes during June 2009 [58]. This step together with existing controversies pertaining to FPG and OGTT, further fuelled differences within the field of diabetes diagnosis. An International Expert Committee appointed by the ADA proposed an HbA1c

diagnostic cut-off value of ≥ 6.5% to efficiently detect diabetes, additionally amending the pre-diabetes category (IFG and IGT) to include an HbA1c in the range of 5.7-6.4% [59].

The HbA1c test assays the attachment of glucose to various amino groups of hemoglobin throughout the 120 day lifespan of an erythrocyte, and thus reflects a 2-3 month glycemic control condition [60-62]. Moreover, fasting is unnecessary for the ensured accuracy of the test, thereby favoring the implementation of HbA1c [32]. In contrast, HbA1c has several limitations (to be discussed in more detail in section 1.3.3). Here principal issues include the lack of standardization and large global inconsistencies [32]. In light of this the WHO remained sceptical by indicating that the role of HbA1c in the effective diagnosis of diabetes was not established enough, thereby refuting its use as an official diagnostic test [56]. The resulting disputes regarding HbA1c's implementation encouraged investigation into its utility, sensitivity and specificity compared to existing glucose-based screening tests [63-66].

1.3.2.3 Comparisons between the diagnostic yields of each test

Studies investigating differences between diagnostic tests generally confirmed a reasonable agreement of HbA1c with FPG and OGTT [67, 68]. Although the performance of HbA1c was similar to that of FPG and OGTT, a cause of concern was that the HbA1c cut-off points employed differed between studies [49]. For example, in a study analyzing the HbA1c diagnostic utility it was deduced that a cut-off as high as 6.5% resulted in the lowest accuracy of diabetes detection [69]. In fact, it detected less than 33% of individuals with undiagnosed diabetes versus FPG diagnostic thresholds [70]. Thus although the HbA1c threshold of 6.5% yields high specificity, its sensitivity is meagre and considerably limiting [62, 71].

The OGTT, although more cumbersome than FPG and HbA1c, can identify a greater number of diabetic individuals than either of the other tests alone [49]. FPG has less variability and increased reproducibility compare to OGTT, but it is known to be influenced by psychological stress and the duration of fasting [72, 73]. Moreover, OGTT and FPG only gauge a single moment of glycemia and this might be fallacious due to the chronic and complex nature of hyperglycemia [73].

By contrast, HbA1c is a precise, stable measurement that captures an individual's average glycemic status. Moreover, HbA1c lacks the problem of adherence to stringent fasting conditions required for OGTT and FPG [73, 74]. Furthermore, HbA1c has the lowest intra-individual variability of these three methods [72]. However, HbA1c is strongly influenced by ethnicity and age, and the discrepancies observed in diagnostic yield are most conspicuous with the diagnosis of non-Hispanic whites [49, 75].

1.3.2.4 The predicting efficiency of each diagnostic test

The differences in predicting outcomes are more subtle. Here FPG, OGTT and HbA1c have been shown to be equally efficient at predicting the development of diabetic complications [76]. However, some studies reported HbA1c to have greater sensitivity and specificity for retinopathy and nephropathy [77], and also providing improved predictive value for cardiovascular risk compared to FPG [78].

During 2011 the WHO validated HbA1c as an official diagnostic test to the conventional means of diabetes diagnosis [51]. Here HbA1c was endorsed as an additional and not as an alternative diagnostic test. Although the diabetic threshold value of 6.5% was adopted, the WHO indicates that an HbA1c level below 6.5%

does not exclude the diagnosis of diabetes. Moreover, they indicate (in contrast to the ADA) that insufficient evidence revokes any formal endorsement for the interpretation of HbA1c values below 6.5% [51].

It must be noted that discordances discussed are most likely due to tests reflecting different facets of glucose homeostasis, making it difficult to compare diagnostic competences (discussed in [79]).

1.3.2.5 The current criteria for the diagnosis of diabetes

Regardless of the on-going and apparent controversies regarding both the diagnostic tests as well as the cut-off criteria characterizing each of these tests, the 2010 ADA recommendations and the 2011 WHO addendum report allow for the diagnosis of diabetes to be confirmed using either HbA1c, FPG or OGTT [49]. Of note, these guidelines do not endorse one particular test as the preferred method of detection [49]. Cut-off values are continually refined and threshold value discrepancies have significantly improved. Despite such progress the present diagnostic tests remain discordant and the current diagnostic criteria (represented in Table 1.2) retain incongruities that may have negative implications.

	2010 ADA CRITERIA	2011 WHO CRITERIA
OGTT		
Normal:	<7.8 mmol/L	<7.8 mmol/L
IGT:	7.8-10.9 mmol/L	7.8-10.9 mmol/L
Diabetic:	≥11 mmol/L	≥11mol/L
FPG		
Normal:	<5.6 mmol/L	<6.1 mmol/L
IFG:	5.6-6.9 mmol/L	6.1-6.9 mmol/L
Diabetic:	≥7.0 mmol/L	≥7.0 mmol/L
HbA1c		
Normal:	<5.7%	Not specified
Pre-diabetic:	5.7-6.4%	Not specified
Diabetic:	≥6.5%	≥6.5%

Table 1.2 The current ADA and WHO diagnostic criteria for OGTT, FPG and HbA1c [49, 54, 56].

1.3.3 Shortcomings of current diagnostic tests

In addition to the apparent discordances discussed above, each screening method contains several idiosyncrasies (technicality and performance) that further impede the efficient detection of diabetes.

1.3.3.1 The oral glucose tolerance test

The OGTT is often referred to as the gold standard for the detection of diabetes (reviewed in [80]). It should be remembered, however, that it acquired gold standard status not because of its efficiency, but because of its longstanding application in a range of different studies (reviewed in section 1.3.2) [81]. However, the OGTT has several methodological and biological shortcomings. For example, it requires the patient to be available for more than 2 hours, has the highest intra-individual variability and the lowest reproducibility of current available tests [72]. Since it has been demonstrated that the rate of glucose absorption differs between male and females, this further limits OGTT's use in clinical practice [82].

1.3.3.2 Glycosylated hemoglobin assay

HbA1c (or A1c) is regularly used to assess an individual's average glucose metabolism (reviewed in [83]). Although it is accredited as a highly specific method for the diagnosis of diabetes, its sensitivity is limiting and it is therefore not as effective in identifying pre-diabetes (reviewed in [84]). The costs of HbA1c assays are also greater than glucose-based tests, making worldwide implementation and uniformity a challenging prospect [73, 74]. Moreover, it is an insufficient detection tool for gestational diabetes (discussed in [49]) and it also does not reflect variability, but only mean glycemia [85]. Various factors can lead to misinterpretation of HbA1c assay results, including certain hemoglobinopathies, iron deficiency, ageing, ethnicity and antiretroviral drugs (ARVs) (reviewed in [49, 59, 73]). These factors significantly hamper the expediency of the HbA1c test, especially in countries where the prevalence of such comorbidities is high. For example, South Africa is a distinctively multi-ethnic country with the highest global prevalence of HIV and AIDS [59, 86]. Indeed, an analysis performed on the application and utility of HbA1c within the South African setting concluded that it should not be used for the diagnosis of diabetes in this instance [74].

1.3.3.3 The fasting plasma glucose test

The fasting plasma glucose test is a simple, inexpensive and standardized tool that is implemented on a frequent basis (reviewed in [49]). However, it fails to indicate daily glycemic fluctuations as it reflects only a single facet of glucose metabolism. Both the fasted and postprandial states are entirely excluded as possible role players when employing this test (discussed in [87]). This limitation can result in a lack of reproducibility and may result in day-to-day variation of results [72]. Additionally, certain factors such as pre-analytical stability [88, 89], and the

patients' stress and activity levels may interfere with the precision of FPG (reviewed in [73]).

It is clear from reviewing the current status of diabetes diagnosis (sections 1.3.2 and 1.3.3), that the investigation into a new diagnostic tool for the earlier, equally sensitive and more efficient detection of type 2 diabetes (with fewer shortcomings and complexities) would be a significant advance. This is the primary objective of this thesis and therefore the remainder of this review represents, in detail, the thought process undertaken to successfully conceptualize and investigate a potentially novel diagnostic tool for the detection of type 2 diabetes.

1.4 The etiology of type 2 diabetes

The best place to begin our investigation is with an exposition of the three metabolic disturbances characteristic of diabetes, i.e. they are hyperlipidemia, insulin resistance and compensatory hyperinsulinemia, and hyperglycemia [90].

1.4.1 Hyperlipidemia

Insulin usually stimulates increased uptake and subsequent storage of glucose (stored as glycogen in liver and muscles, and as triglycerides in adipose tissue), along with the inhibition of lipolysis and glycogenolysis in adipose tissue and the liver, respectively [91]. Insulin resistance can be defined as the reduced responsiveness of the adipose, muscle and liver cells to the effects of insulin [92].

Hyperlipidemia usually presents with elevated blood levels of non-esterified fatty acids (NEFAs) and triglycerides (reviewed in [90]). NEFAs play a large role in the development of insulin resistance [93-95] and several theories exist to explain how free fatty acids can induce decreased insulin sensitivity [96].

For example, Randle *et al.* (1963) proposed that insulin resistance associated with obesity is due to elevated fat oxidation [97-99]. The glucose-fatty acid cycle hypothesis is based on increased acetyl-CoA/CoA and NADH/NAD$^+$ ratios decreasing the activity of pyruvate dehydrogenase (PDH) which results in elevated intracellular citrate levels. Increased citrate level inhibits phosphofructinase (PFK), a rate-limiting glycolytic enzyme, leading to an accumulation of glucose-6-phosphate (G-6-P). This in turn results in the inhibition of hexokinase II activity, causing elevated intracellular glucose concentrations and decreased glucose uptake through glucose transporter 4 (GLUT4) [97](Figure 1.3 A).

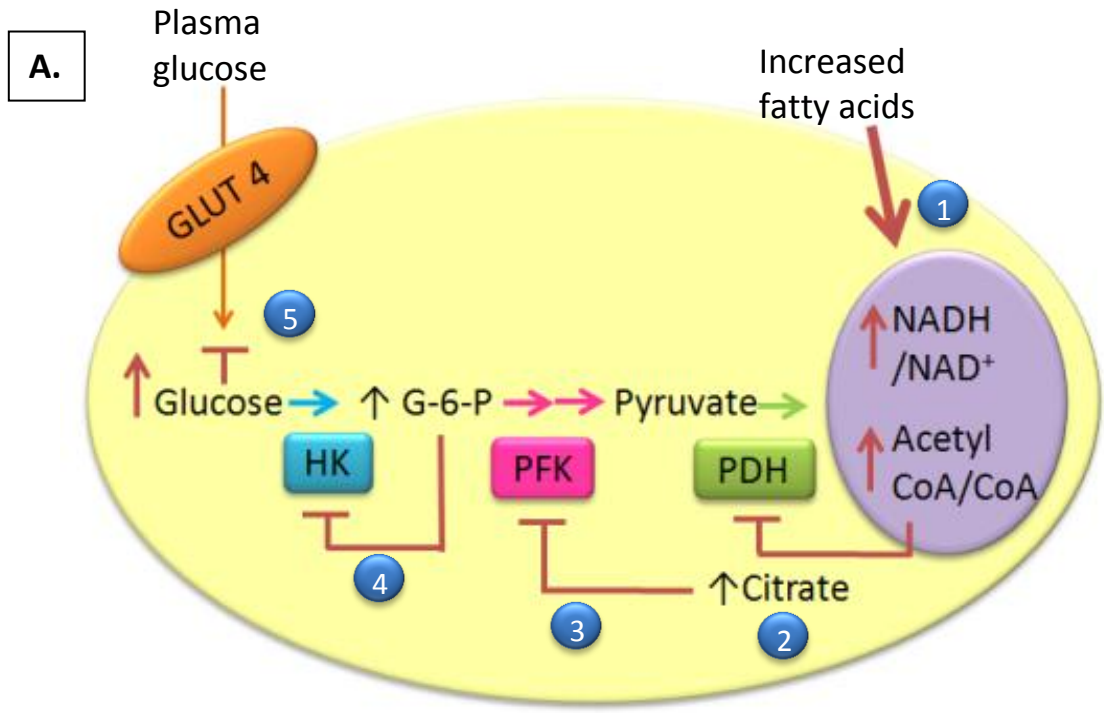


Figure 1.3 Examples of proposed mechanisms for hyperlipidemia-induced insulin resistance. **A:** The Randle hypothesis as described in the text (1). Elevated fat oxidation leads to increased acetyl-CoA/CoA and NADH/NAD$^+$ ratios inactivating PDH, (2) resulting in increased citrate levels (3). Inhibition of PFK leads to an accumulation of G-6-P, which results in the inhibition of hexokinase II, (4) increasing glucose concentrations and (5) decreasing glucose uptake via GLUT4.

Numerous studies have since challenged the Randle hypothesis [94, 100-102]. For example, an opposing theory by Shulman *et al.* (2000) suggests that the increase in

fatty acid metabolites (diacyglycerol, fatty acyl CoAs, ceramide) disturb the normal functioning of the insulin signaling pathways by activating protein kinase C (PKC) θ [91]. Here PKC θ initiates a serine/threonine cascade leading to the subsequent phosphorylation of serine residues on insulin receptor substrate-1 (IRS-1). The successive association of IRS-1 with phosphatidylinositol 3-kinase (PI3K) is dependent on the phosphorylation of threonine amino groups. Therefore serine-phosphorylated IRS-1 alters the functional properties of the insulin signaling pathway, resulting in decreased GLUT4 translocation to the sarcolemma and attenuated glucose uptake into the cell (Figure 1.3 B) [91, 103]. Several rodent and human studies support this theory [101, 102, 104, 105].

Increased fatty acid metabolites can also alter insulin signaling pathways through an alternative mechanism [90]. The PI3 kinase/Akt-1 pathway plays a pivotal role in ensuring efficient insulin action, and the phosphorylation and subsequent activation of Akt-1 is essential for the regulation of GLUT4 [106]. The activation of Akt-1 is dependent on the production and phosphorylation of phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P3) [107]. NEFAs, however, can act as natural ligands for peroxisome proliferator-activated receptor (PPAR), a transcriptional modulator which is capable of up-regulating the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [108]. The subsequent up-regulation of PTEN results in the dephosphorylation of PtdIns(3,4,5)P3 and therefore promotes the loss of insulin sensitivity through the inhibition of Akt-1 activation (Figure 1.3B) [90].

An additional fatty acid-induced mechanism that promotes insulin resistance is the attenuation of insulin receptor (IR) gene expression [109]. It is postulated that palmitate can inhibit IR expression, thus decreasing IR protein levels in insulin-

dependent target cells [109]. Decreased IR gene expression is possibly due to PKC phosphorylation (various isoforms) (Figure 1.3B) [110].

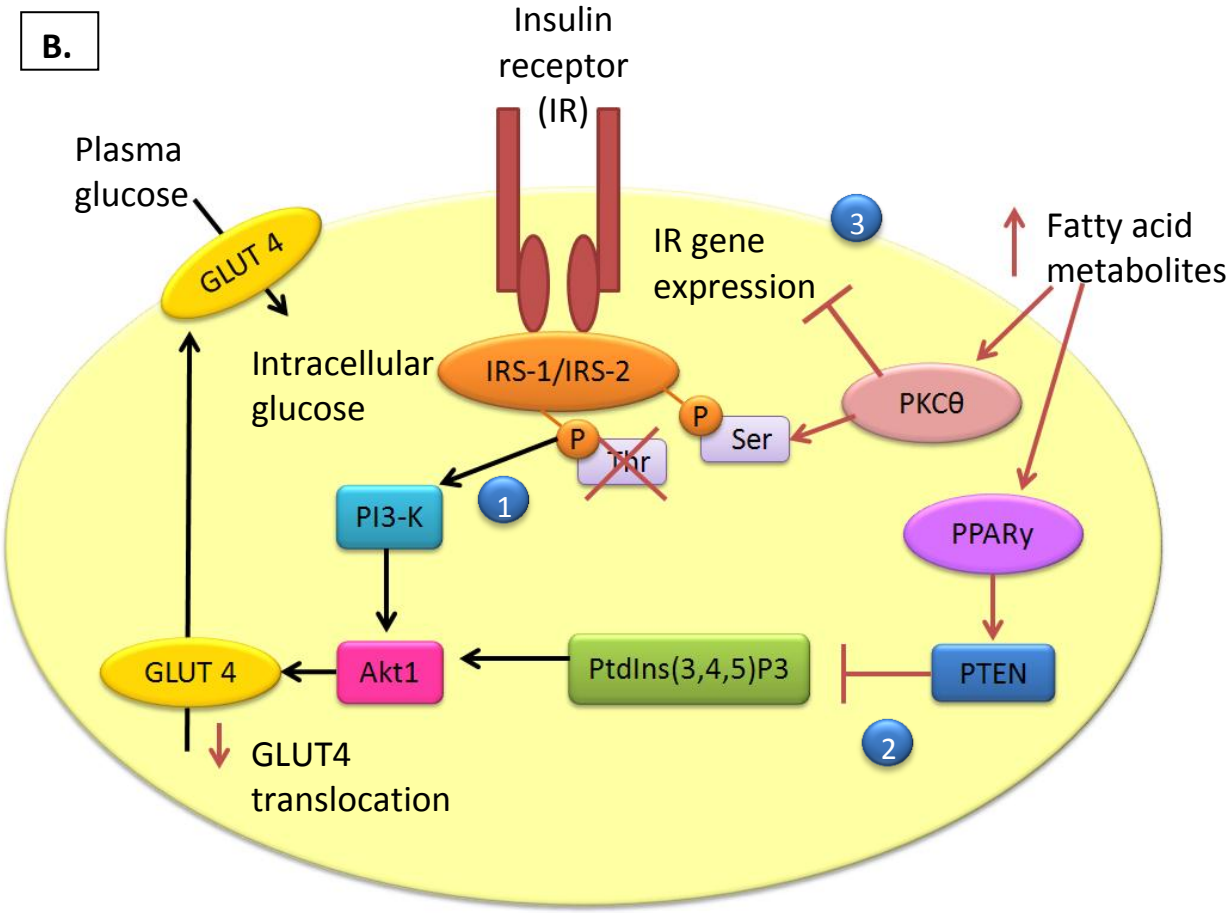


Figure 1.3 B: Three additional examples of proposed mechanisms by which fatty acid metabolites reduce insulin sensitivity as described in the text (1). PKCθ leads to the phosphorylation of serine instead of threonine residues on IRS-1, disturbing the insulin signaling pathway and resulting in decreased GLUT4 translocation. (2). Up-regulation of PTEN results in the dephosphorylation of PtdIns(3,4,5)P3 and the inhibition of Akt-1 activation (3). Fatty acid-induced attenuation of IR gene expression. PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; G-6-P, glucose-6-phosphate; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; IRS-1, insulin receptor substrate-1; (PtdIns(3,4,5)P3), phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; IR, insulin receptor.

The discussed examples of mechanisms by which hyperlipidemia induces insulin resistance make it clear that NEFAs play a significant role (various mechanisms) in

altering cellular insulin signaling pathways and thereby contribute to insulin resistance and compensatory hyperinsulinemia [90].

1.4.2 Insulin resistance and compensatory hyperinsulinemia

It should be noted that, in addition to the lipid-induced insulin resistance already discussed, the pathophysiology of insulin resistance can also be caused by mitochondrial dysfunction, glucocorticoids, inflammation, oxidative stress (refer to section 1.5) and the hexosamine biosynthetic pathway (HBP) (to be discussed in section 1.7)[111-113]. Moreover, evidence shows that surplus glucose itself can play a significant role in the development of insulin resistance through the down regulation of 5′-AMP-activated protein kinase (AMPK), a key enzyme responsible for elevated GLUT4 translocation (especially during exercise) [114].

Insulin resistance may precede full-blown diabetes by more than a decade (discussed in [90]). This condition is therefore of a progressive nature and as the severity of insulin resistance increases, pancreatic beta cells are required to secrete increased amounts of insulin in an attempt to maintain glucose homeostasis [115]. During the early stages of insulin resistance such compensatory increases in insulin concentrations are adequate to maintain normal glucose homeostasis [116, 117]. This in turn results in a normoglycemic hyperinsulinemic state, i.e. individuals are insulin resistant but display fasting plasma glucose levels in the normal range [116, 118, 119]. It must be noted that mild increases in blood glucose levels do occur, but due to concentrations remaining within the normal classification range, this typically remains unnoticed (discussed in [115]).

Due to the progressive loss of insulin sensitivity, the ability of the pancreas to secrete the large amounts of insulin required to maintain glucose homeostasis

becomes hampered (discussed in [120, 121]. Moreover, hyperinsulinemia has an independent and pathogenic function in the development of insulin resistance [92, 122] which leads to a pre-diabetic (IGT/IFG) setting that is initially characterized by hyperinsulinemia and hyperglycemia (discussed in [123, 124]). Due to prolonged insulin resistance, hyperinsulinemia and glucolipotoxicity, the function of pancreatic beta cells begin to decline, and although still elevated, insulin levels begin to descend [115, 125].

The pre-diabetic states (IFG and IGT) are heterogeneous, and although isolated IFG and IGT are both insulin resistant conditions [126], differences occur with regard to the exact site of decreased insulin sensitivity. Here IFG principally presents as hepatic insulin resistance, while IGT is predominantly characterized by muscle insulin resistance [127, 128].

IFG and IGT also differ in their pattern of insulin release. The kinetics of insulin secretion (biphasic) is vital to understand the implications of insulin resistance [129]. The first phase has a duration of only 15 minutes and is a rapid release of insulin (peaks at 2-4 minutes in response to a hyperglycemic stimulus)[130]. First phase insulin is pre-formed and stored in granules within the beta cell [130]. The second phase of insulin secretion is gradual and increases progressively for up to 3 hours [129]. Individuals with isolated IFG experience a defect in their early-phase insulin response. However, IGT patients exhibit decreased first phase insulin secretion and a major deficiency in the second phase of insulin release [127]. This helps to elucidate variances in glucose levels between the two pre-diabetic states. Glycogenolysis in the liver together with a deficit in the early phase of insulin release result in excess hepatic glucose output, thereby leading to the elevated fasting plasma glucose levels observed in IFG [127]. By contrast, decreased muscle

glucose uptake together with blunted late phase insulin secretion is responsible for the post-prandial hyperglycemia (PPG) during IGT [127]. Individuals with combined IFG/IGT experience both hepatic and muscle insulin resistance [127].

During the latter stages of insulin resistance, the steady decline in insulin secretion correlates with further elevations in hyperglycemia, raising glucose concentrations into the diabetic glucose range [123]. Type 2 diabetes is characterized by severe hyperglycemia and significantly reduced insulin levels [131]. This translates into diminished insulin secretion that together with the insulin sensitivity deficiency results in a severe level of glycemic dysregulation [132]. In patients with established overt diabetes, complete beta cell failure takes place and such individuals therefore require insulin therapy [120].

1.4.3 Hyperglycemia

To summarize, increased insulin resistance, compensatory hyperinsulinemia, beta cell dysfunction and the subsequent decrease in insulin secretion lead to aberrant glucose metabolism. Diminished insulin sensitivity in adipose tissue impairs the suppression of lipolysis, elevating fatty acid metabolites and thereby further impairing insulin resistance (reviewed in [115, 133]). Hepatic insulin resistance results in decreased glycogen production as well as a defect in the inhibition of gluconeogenesis [134]. Moreover, a critical consequence of insulin resistance is reduced insulin-mediated muscle glucose uptake [132, 135]. Collectively, this contributes toward abnormal glucose homeostasis and various severities of hyperglycemia (IGT/IFG/Diabetes) [115, 120].

Glucose uptake is regulated by both insulin-dependent as well as insulin-independent mechanisms [132]. Due to the hydrophilic nature of glucose, specific glucose transport proteins are used to facilitate glucose uptake [132]. Here GLUT1 and GLUT4 are the two major isoforms responsible for whole body insulin-stimulated glucose disposal [132]. GLUT1 is predominantly expressed in erythrocytes, kidney and colon, while expressed at very low levels in muscle, liver and adipose [132, 136]. GLUT4 is thus the major transporter responsible for insulin-stimulated glucose clearance by the major disposal sites (i.e. muscle and adipose) [136, 137]. GLUT 2 is the major transporter in the liver [137]. More recently, sodium-glucose co-transporters (SGLTs) have been identified to play an important role in glucose transport during hyperglycemia [138]. However, GLUT4 translocation is reduced with insulin resistance [139] (as discussed in section 1.4.1), and therefore insulin-dependent glucose uptake is decreased [132].

The failure in efficient glucose uptake by the liver, muscle and adipose results in elevated blood glucose concentrations (hyperglycemia) (as discussed in sections 1.4.2 and 1.4.3). Consequently, glucose uptake is increased through insulin-independent mechanisms (reviewed in [140]). This therefore augments glucose metabolism in insulin-insensitive tissues [140]. Insulin-independent glucose clearance occurs due to plasma glucose exerting a mass action effect (discussed in [132]). Various other GLUT isoforms are also involved in the facilitative diffusion of glucose down its concentration gradient [132]. After uptake, increased glycolytic flux triggers greater oxidative stress production thereby leading to downstream metabolic defects. This will now be discussed in more detail in the sections to follow.

The exact mechanisms of hyperlipidemia, hyperinsulinemia and hyperglycemia are still not completely clear and there is still some controversy with respect to the role and significance of these mechanisms in the context of type 2 diabetes pathophysiology. However, it is evident that complex relationships exist between these three metabolic abnormalities. Moreover, when summarizing the above discussion into a schematic diagram and presented together in Figure 1.4, the severity of the situation is further emphasized by their interactions leading to a positive feedback loop which further exacerbates metabolic dysregulation (refer to Figure 1.4).

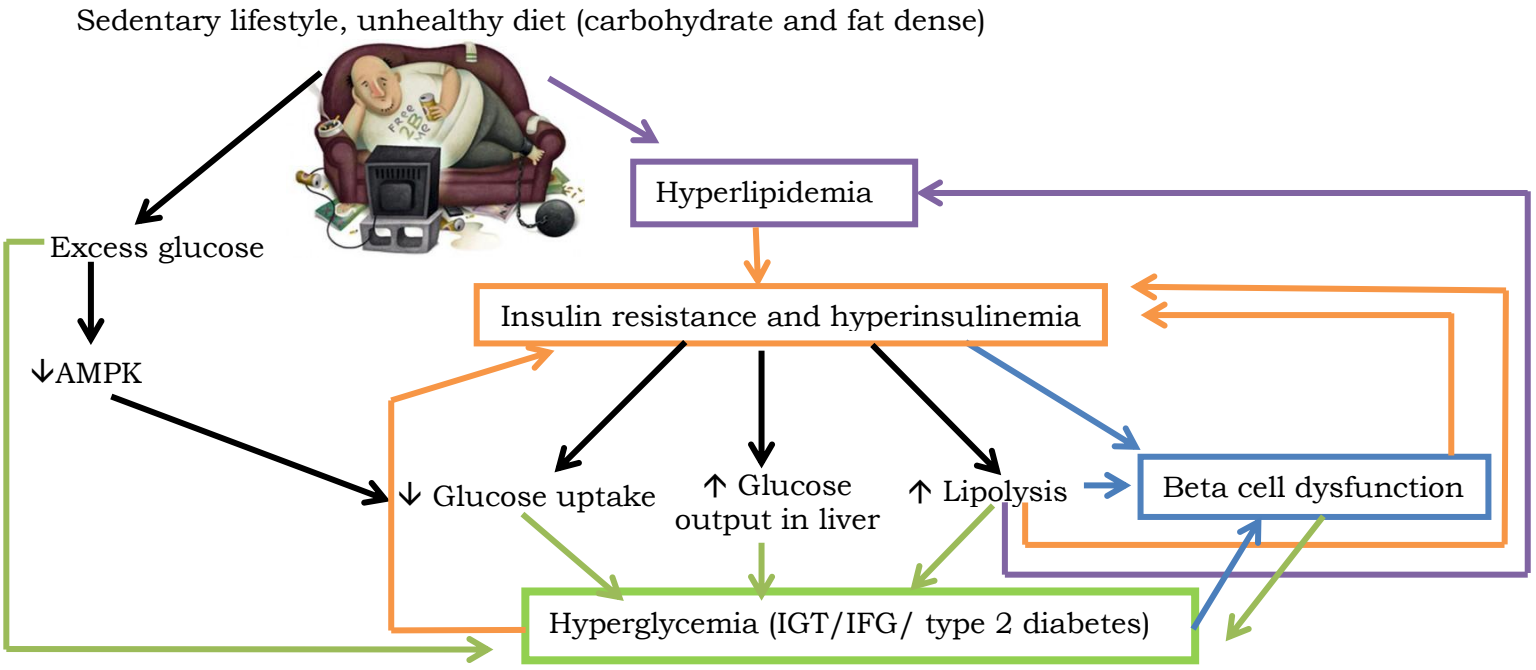


Figure 1.4 Metabolic abnormalities of diabetes are interlinked and lead to a self-perpetuating vicious cycle. AMPK, 5′-adenosine monophosphate activated protein kinase; IGT, impaired glucose tolerance; IFG, impaired fasting glucose.

1.5 Hyperglycemia-induced oxidative stress production

The generation of oxidative stress occurs due to an imbalance between rates of oxidant production and its scavenging [141]. Hyperglycemia increases glucose

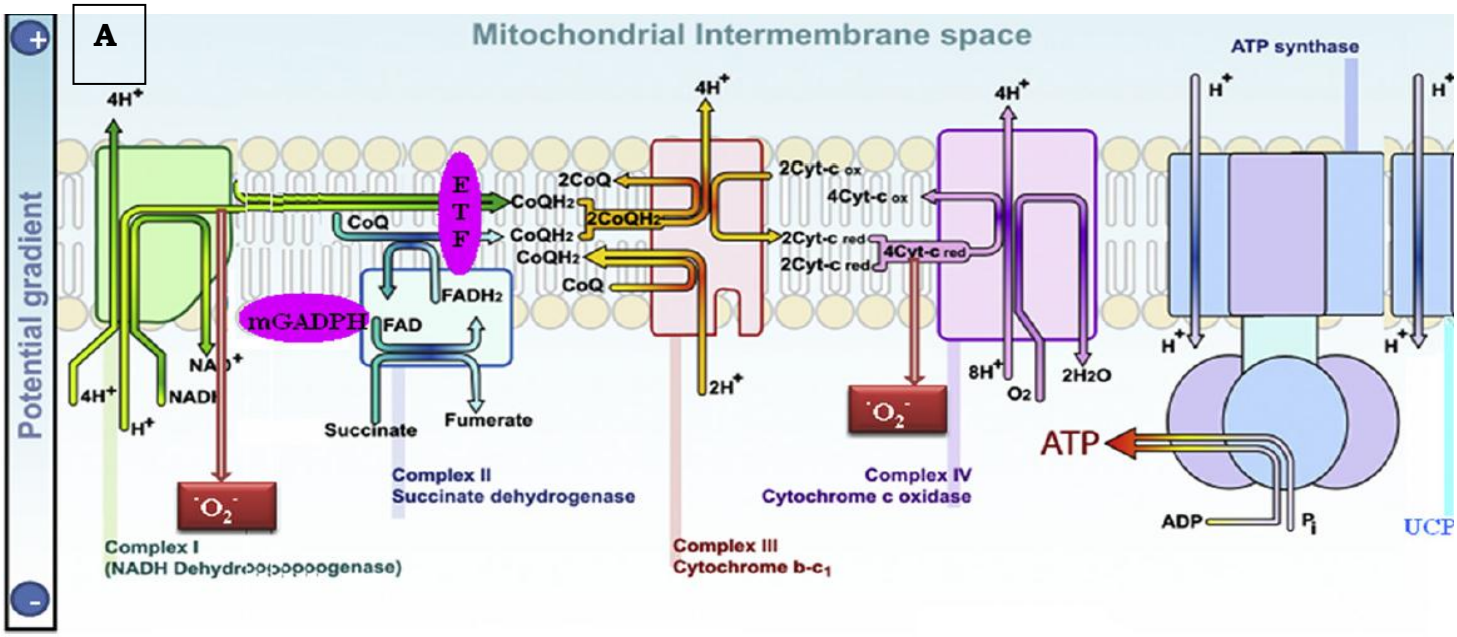
levels which feed into metabolic pathways and this in turn a) enhances oxidative stress and b) impairs antioxidant defences [142]. Moreover, such generated oxidative stress is now recognized as a major contributor to several diabetic complications that include micro- and cardiovascular pathologies (reviewed in [140, 143, 144]).

1.5.1 Mitochondrial superoxide production

The mitochondrion is the principal source of hyperglycemia-induced oxidative stress (reviewed in [145]). Under normal physiological conditions the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (mitochondrial electron transport chain) generate energy through pyruvate oxidation. Electron transfer takes place through mitochondrial complexes I, III and IV, and produces a proton gradient by moving protons into the intermembrane space [146]. Subsequently, protons re-enter the mitochondrial matrix thereby driving ATP synthase and mitochondrial ATP production [142]. Uncoupling proteins can control the magnitude of the proton gradient by converting the extruded protons into heat, thereby ensuring that the ATP production rate remains constant (Refer to Figure 1.5A). In this manner the electron transport chain can precisely regulate ATP levels under physiological conditions [147].

Mitochondrial superoxide ($O_2^{\bullet-}$) is usually produced at complex I and III, which is then scavenged by superoxide dismutase (SOD) and glutathione peroxidase (GSPx) [148]. More recently, complex II has also been associated with superoxide production [149]. However, with hyperglycemia increased glycolytic flux results in elevated glucose-derived pyruvate entering the TCA cycle [146]. More pyruvate is therefore oxidized, which augments NADH and $FADH_2$ levels and thus increases the

intermembrane proton gradient until a threshold is reached [150]. Under these conditions a blockage at complex III results with a corresponding accumulation of electrons at coenzyme Q (CoQ) [151]. Surplus electrons are accordingly donated to oxygen, thereby overproducing mitochondrial superoxide (represented in Figure 1.5B) (reviewed in [152]). Increased free fatty acid (FFA) oxidation leads to greater mitochondrial ROS production (specifically superoxide) and activation of UCPs [153]. The resulting increase in proton export will eventually cause mitochondrial uncoupling (reviewed in [154]). This hypothesis confirms that superoxide formation is the major source of hyperglycemia-induced mitochondrial ROS and therefore most relevant to our study due to its specific downstream detrimental effects (to be described in section 1.6). However, it should be noted that superoxide is not the only ROS type produced by mitochondria (discussed in [153, 155]). For example, manganese superoxide dismutase (MnSOD) reduces superoxide to hydrogen peroxide (H_2O_2) which is usually detoxified by catalase into H_2O and oxygen (reviewed in [156]). However, catalase activity is inhibited under hyperglycemic conditions and thus H_2O_2 can be converted into various alternative ROS forms [153].



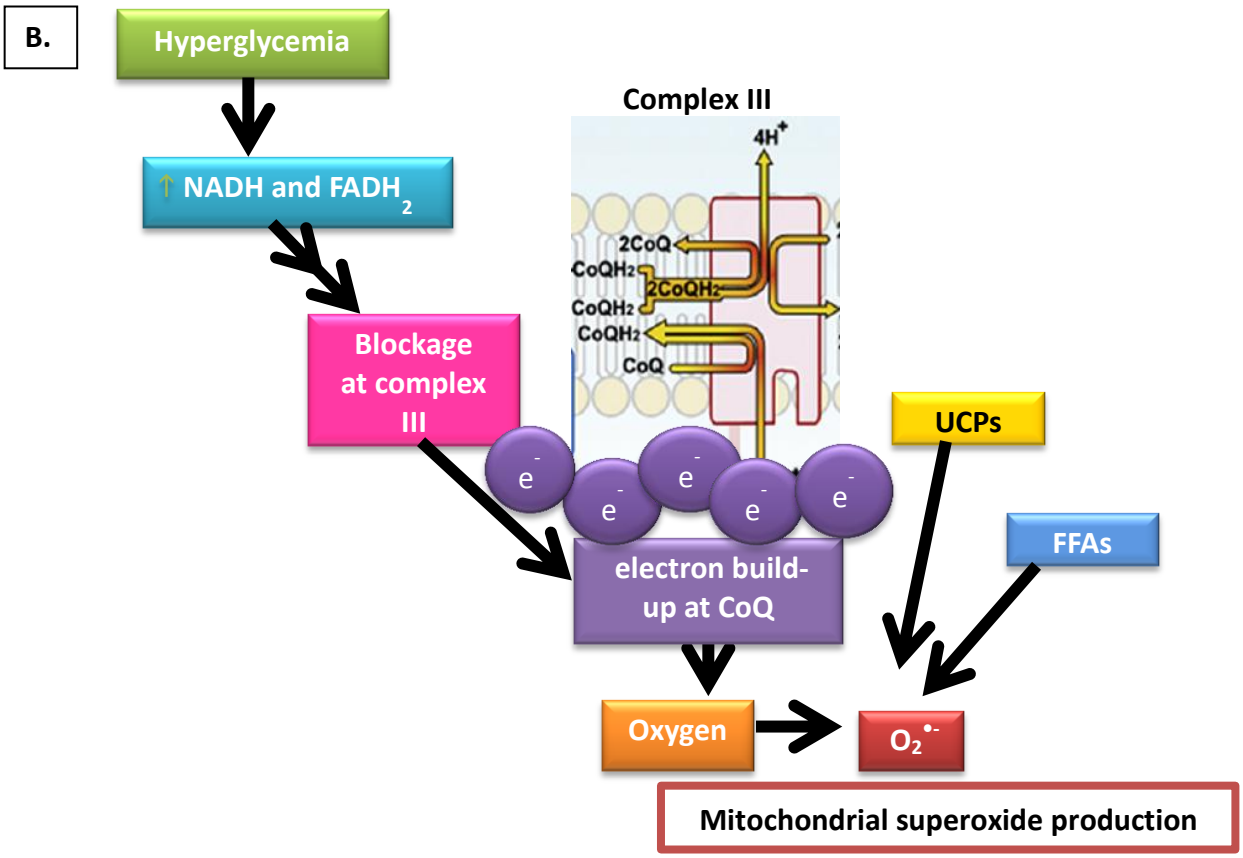


Figure 1.5 Mitochondrial electron transport chain under physiological versus hyperglycemic conditions. A: Under normal conditions a well-controlled proton gradient drives the production of ATP via ATP synthase. B: During conditions of hyperglycemia, increased glycolytic flux increases the availability of electron donors (NADH, $FADH_2$) entering the mitochondrial ETC. The voltage gradient across the intermitochondrial membrane is up-regulated leading to defects in electron transfer and ultimately the production of superoxide. FAD: Flavin adenine dinucleotide (oxidized),$FADH_2$: Flavin adenine dinucleotide (reduced), NAD^+ :Nicotinamide adenine dinucleotide (oxidized), NADH: Nicotinamide adenine dinucleotide (reduced), UCPs: Uncoupling proteins, CoQ: Coenzyme Q,$O_2^{\bullet-}$:superoxide.

1.5.2 Additional sources of oxidative stress

Although the mitochondrion is the primary source of hyperglycemia-induced oxidative stress, smaller amounts of oxidative stress are produced through other metabolic pathways (reviewed in [146]). For example, increased glucose flux can lead to oxidant production via the non-enzymatic glycation of proteins and the enhanced activity of aldose reductase and PKC (reviewed in [157]). These

alternative metabolic pathways are various off-shoots of the glycolytic pathway and will be discussed in detail in section 1.6.

1.6 Hyperglycemia-mediated mitochondrial superoxide production activates alternative glucose-utilizing pathways

The majority of glucose that enters the cell is typically metabolized via glycolysis. However, additional non-oxidative glucose metabolizing pathways also play a role in the metabolism of glucose under normal conditions [146]. During hyperglycemia flux through alternative glucose-utilizing pathways increases, thereby diverting excess glucose supply [143]. Up-regulation of four of these pathways is strongly implicated in hyperglycemia-induced vascular damage and various diabetes-related pathologies [158, 159].

There is a unifying hypothesis that the hyperglycemia-induced activation of non-oxidative glucose pathways is triggered by a single upstream mechanism: the overproduction of mitochondrial superoxide [152, 160]. An important aspect of such superoxide-induced up-regulation is the elevation of poly(ADP-ribose) polymerase (PARP) activity, a nuclear enzyme involved in DNA repair [161, 162]. Here the proposal is that overproduction of mitochondrial superoxide (described in section 1.5.1) results in the breakage of DNA strands, thereby inducing the activation of PARP [163]. PARP produces polymers of ADP ribose, leading to its targeted modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme [164]. GAPDH activity decreases as a result of such a modification (Figure 1.6) [142]. This in turn leads to the accumulation of glycolytic intermediates upstream of GAPDH, resulting in the up-regulation of five non-oxidative glucose utilizing pathways, namely the pentose phosphate pathway (PPP),

polyol pathway, the formation of advanced glycation end-products (AGE), activation of PKC, and the HBP (Figure 1.6) (reviewed in [143, 146, 153]). The main focus of this study is the assessment of HBP flux in response to various degrees of hyperglycemia (Figure 1.6).

Of note, although the up-regulation of PKC, AGE, polyol and HBP is activated primarily through the hyperglycemia-induced superoxide pathway discussed [152], higher substrate availability during hyperglycemia can also increase pathway flux independent of GAPDH inhibition.

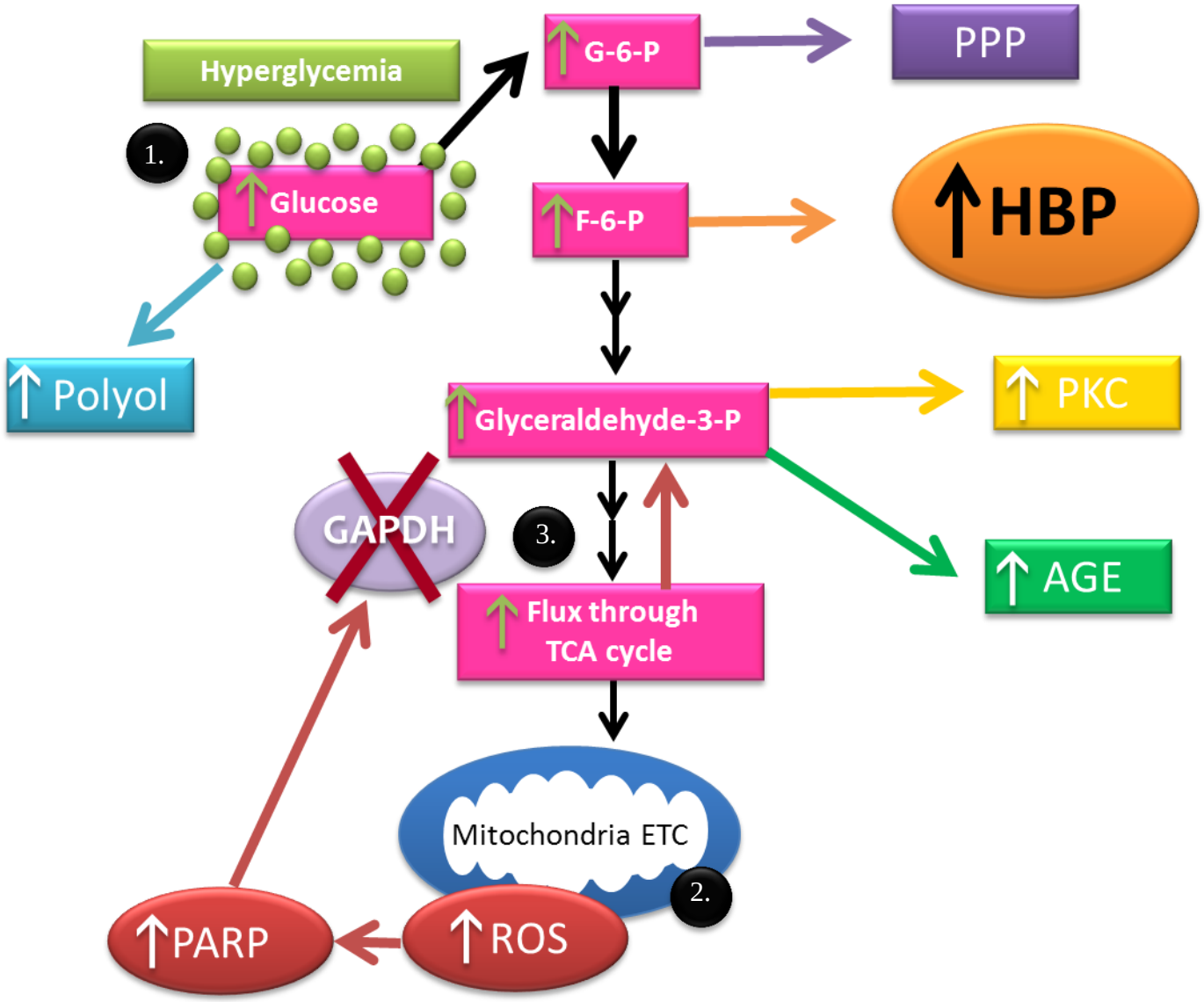


Figure 1.6 Hyperglycemia-induced mitochondrial superoxide production inhibits GAPDH and leads to the up-regulation of five alternate glucose metabolizing pathways. (1) Hyperglycemia increases glycolytic flux and elevates mitochondrial ROS production → (2). Superoxide increases PARP activity which inhibits GAPDH activity → (3). Upstream metabolites accumulate and glucose flux is increased via alternative glucose-utilizing pathways (→PPP), (→HBP), (→PKC), (→ AGE), (→ Polyol) G-6-P:Glucose-6-phosphate; F-6-P:Fructose-6-phosphate; TCA: Tricarboxylic acid; ETC: Electron transfer chain; PARP: poly(ADP-ribose) polymerase GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AGE: Advanced glycation end-products; PPP: pentose phosphate pathway, PKC: Protein kinase C; HBP: Hexosamine biosynthetic pathway.

1.6.1 Pentose phosphate pathway (PPP)

Of the non-oxidative pathways that are up-regulated under hyperglycemic conditions, increased PPP flux is not considered harmful since it generates NADPH (and thereby contributes to intracellular sources of reducing equivalents) [165, 166]. This pathway consists of both an oxidative and non-oxidative phase [167]. The oxidative phase results in the production of NADPH through the conversion of glucose 6-phosphate to ribulose-5-phosphate (Figure 1.7) (discussed in [168]). As discussed, NADPH plays a key and beneficial role in glucose metabolism, as the scavenging activity of various antioxidants are dependent on its availability [140]. This is therefore a likely way how the PPP plays a protective role during oxidative stress conditions.

Transketolase is the rate-limiting enzyme of the non-oxidative PPP (Figure 1.7) [142]. Under normal conditions it converts pentose phosphates into glycolytic intermediates, however, it can also perform the reverse reaction [169]. Thus transketolase is able to decrease the concentration of glycolytic intermediates (fructose-6-phosphate and glyceraldehyde-3-phosphate) [146]. Interestingly, PPP up-regulation can divert glucose flux away from the four “harmful” non-oxidative pathways and as a result has sparked several research ventures that investigate this potential beneficial role [170]. Although the advantages of the PPP may not always outweigh the detrimental effects of the four non-oxidative pathways, it provides great promise for a future therapeutic target (e.g. transketolase activators) (discussed in [146]). Thus the PPP provides an opportunity for a regulated metabolic reconfiguration, thereby serving as the cells’ defense mechanism in response to a) increased oxidative stress and b) the up-regulation of the following four “damaging” pathways [166].

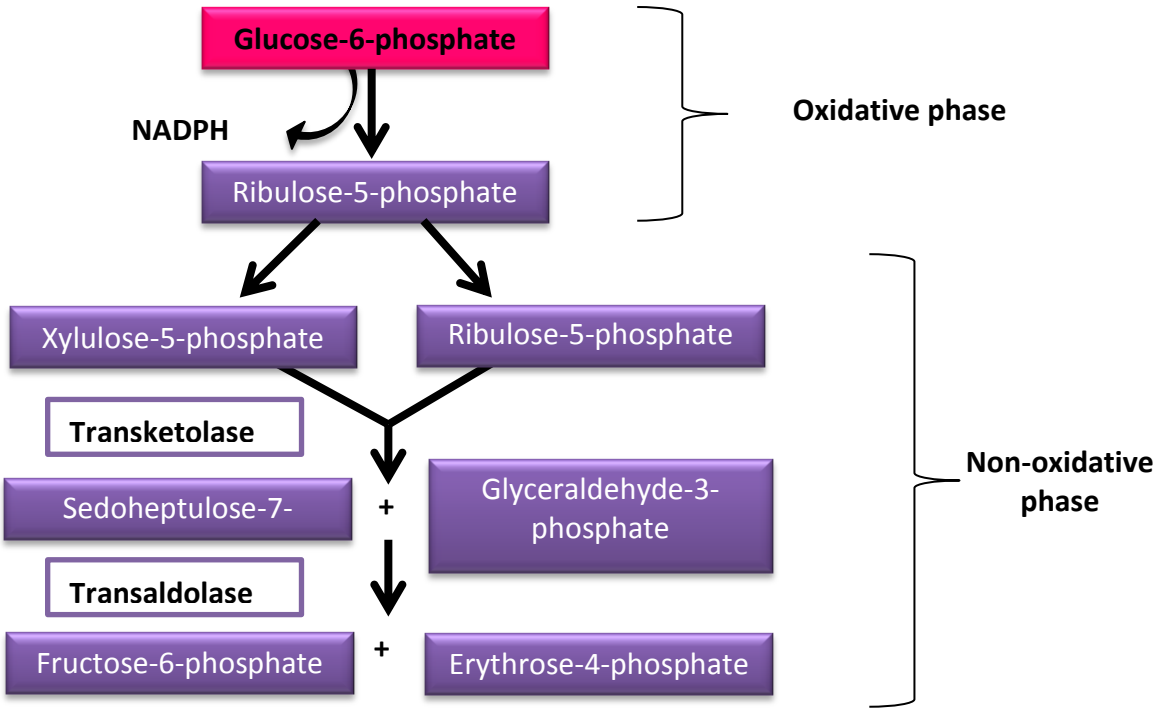


Figure 1.7 Schematic representation of the pentose phosphate pathway.

1.6.2 Polyol pathway

During normoglycemia ~3% of glucose enters the polyol pathway (discussed in [153]). Here aldose reductase typically functions to reduce aldehydes that are toxic to the cell and thereby inactivate sugar alcohols (polyols) (Figure 1.8) [171]. However, during hyperglycemic conditions aldose reductase additionally reduces glucose (with reducing power of NADPH) to sorbitol [171]. Aldose reductase is present in cell types vulnerable to the effects of hyperglycemia, including the retina, glomerulus, nerves and vascular cells (tissues widely associated with damage in type 2 diabetes) [172]. Sorbital dehydrogenase thereafter oxidizes sorbital into fructose, with the loss of NAD^+ (Figure 1.8) [173]. Therefore increased flux through the polyol pathway leads to the depletion of NADPH, an essential cofactor for reduced glutathione (GSH), a key intracellular antioxidant (Figure 1.8) [174]. Thus under conditions of hyperglycemia and oxidative stress reduced NADPH availability aggravates the imbalance between oxidant production and antioxidant activity.

This renders the cell defenseless and even more susceptible to the damaging effects of ROS [146]. Hyperglycemia-induced up-regulation of the sorbital pathway elicits additional negative effects such as decreased myoinositol uptake and an increased production of vasodilatory prostaglandins [146].

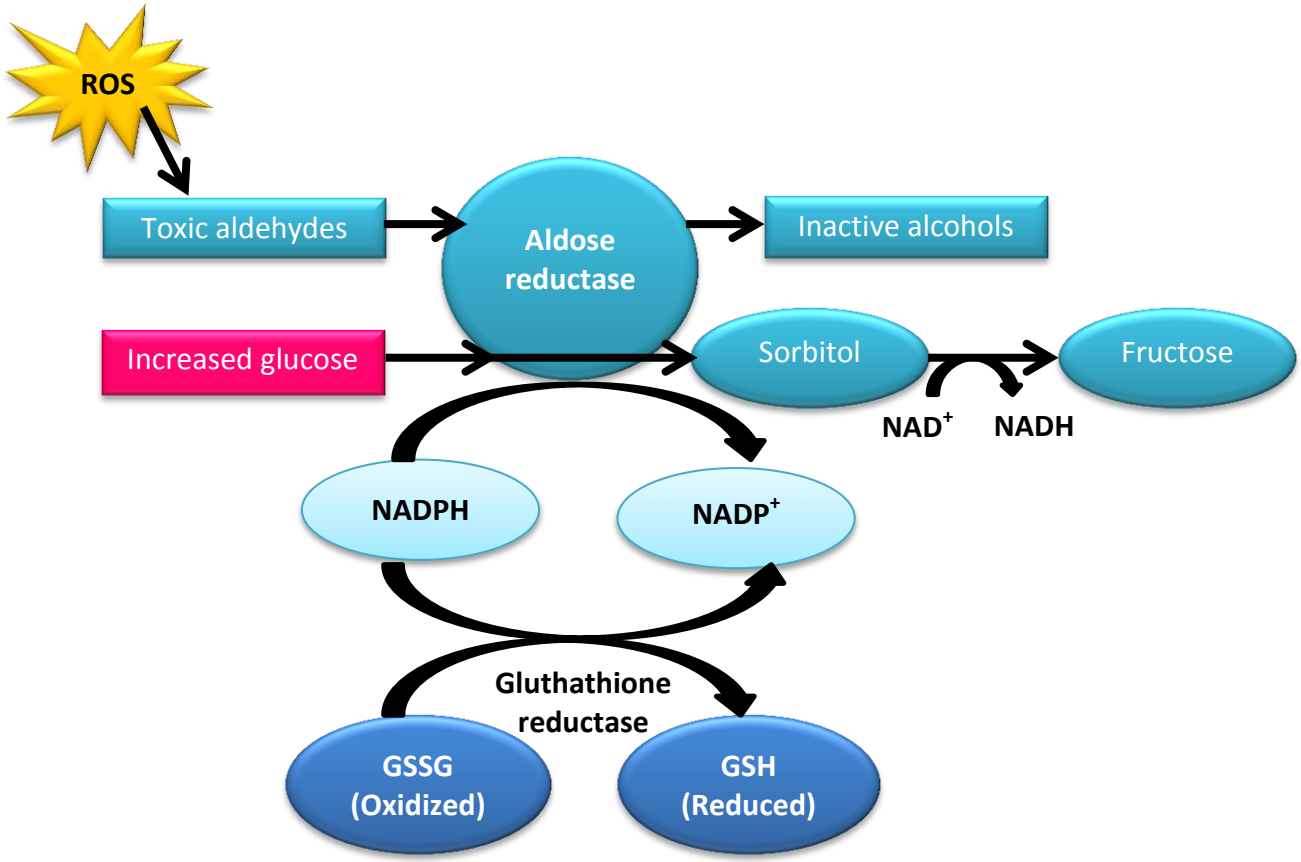


Figure 1.8 Schematic diagram of the polyol pathway (Reproduced from [142]).

1.6.3 AGE formation

Glucose together with other glycating compounds is able to react non-enzymatically to form AGEs [142]. The auto-oxidation of glucose, decomposition of the Amadori (an isomerization rearrangement) product, and the fragmentation of glyceraldehyde-3-phosphate result in the formation of reactive intracellular dicarbonyls: glyoxal, 3-deoxyglucosone and methylglyoxal, respectively (Figure 1.9) [146, 175]. Subsequently, dicarbonyls are able to react spontaneously with amino

groups of proteins to form AGEs (Figure 1.9) [147]. Augmented glucose concentrations and elevated glycolytic flux result in increased AGE production and it is particularly prevalent in the liver, kidneys and erythrocytes of individuals with type 2 diabetes [176].

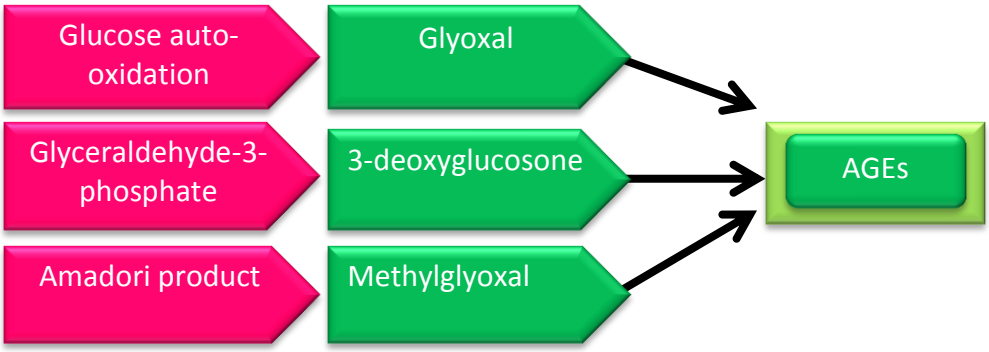


Figure 1.9 Diagram depicting the formation of AGEs.

The formation of AGE precursors harm target cells by three mechanisms, i.e. 1) altering the functional properties of target proteins, 2) interfering with matrix-matrix and matrix-cell interactions, and 3) AGE binding to their cell surface receptors, i.e. receptors of AGE (RAGE) (reviewed in [147, 177]. The latter mechanism results in the generation of intracellular ROS, that can increase the production of the transcription factor NFκ-B and further elevate ROS production [176]. Moreover, RAGE-induced cytosolic ROS can promote the production of mitochondrial superoxide in the kidneys of diabetic rats [178], reinforcing a positive feedback loop of sustained oxidative stress production and its subsequent damage.

1.6.4 Activation of PKC

Excess glucose can directly increase the activation of PKC either through the *de novo* synthesis of diacylglycerol (DAG), activation of phospholipase C or the inhibition of DAG kinase (Figure 1.10) [179, 180]. Moreover, there is some evidence that surplus glucose can indirectly increase PKC levels. One proposed mechanism

is that interactions between AGEs and their receptors can enhance PKC activity [181].

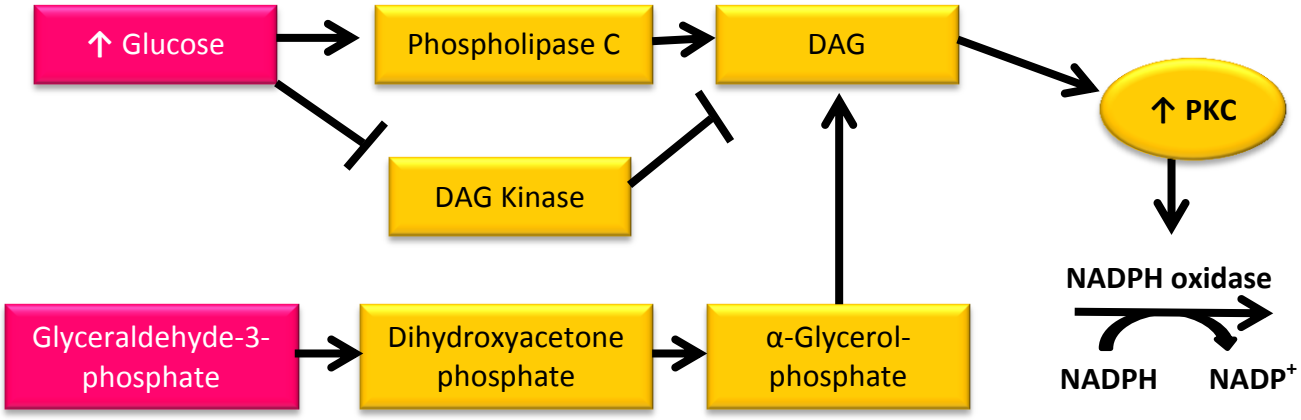


Figure 1.10 Schematic illustrating the activation of PKC (adapted from [140]).

PKC activation can also increase oxidative stress by stimulating the activity of NADPH oxidase (Figure 1.10). This decreases NADPH/$NADP^+$ ratios thereby decreasing NADPH availability and triggering oxidative stress (reviewed in [140]). This can occur by decreased GSH regeneration and subsequent glutathione scavenging activity, and also by the attenuation of catalase activity (H_2O_2 detoxification enzyme) (reviewed in [140]). Augmented PKC activity is also associated with functional changes to vascular cells, the expression of growth factors and alterations of specific basement membrane proteins [153].

1.6.5 Hexosamine biosynthetic pathway (HBP)

The HBP is known to be an overflow pathway of glycolysis as well as a nutrient or fuel sensor that (under normal circumstances) utilizes approximately 3% of the total glucose available to the cell [182, 183]. Glutamine:fructose-6-phosphate aminotransferase (GFAT) is the rate-limiting enzyme of the HBP and responsible for the catalytic conversion of fructose-6-phosphate to glucosamine-6-phosphate [184].

A series of successive reactions result in the post-translational modification of target proteins by addition of an *O*-GlcNAc moiety. During hyperglycemia increased *O*-GlcNAcylation of target proteins is strongly associated with the onset of CVD and insulin resistance [185]. Moreover, the increased production of glucosamine-6-phosphate inhibits the activity of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the PPP [186]. This inhibition ultimately leads to a decrease in NADPH/$NADP^+$ ratios since G6PD activity involves the reduction of $NADP^+$ to NADPH [140]. The HBP therefore plays a role in mitigating the PPP and the beneficial role it plays in boosting intracellular defenses. The decreased availability of NADPH attenuates intracellular antioxidant capacity and further contributes to ROS production (decreased GSH and catalase activity) [140].

1.6.6 Summary of damaging pathways activated by hyperglycemia

There are four major pathways involved in the pathogenesis of diabetic complications [146]. Hyperglycemia leads to the production of mitochondrial superoxide which inhibits GAPDH, resulting in the accumulation of upstream glycolytic intermediates (Figure 1.6). The various intermediates are shunted into four glucose-utilizing pathways (polyol, AGE, PKC and HBP) that can lead to cellular damage. Increased flux through such pathways lead to higher ROS production and the weakening of antioxidant defenses, in addition to various other damaging effects. Intracellular ROS production is additionally implicated in further perpetuating mitochondrial superoxide production [187]. Moreover, HBP activation is associated with the development of insulin resistance and several vascular complications associated with diabetes (to be discussed in more detail in section 1.8) [188]. The full impact of these pathways as well as the exact mechanisms linking their upregulation to the progression of diabetic complications is not

completely understood. However, it is clear that the interactions and effects of these pathways are interlinked and further contribute and aggravate the self-perpetuating vicious cycle of metabolic defects illustrated in Figure 1.4. Thus in turn this leads to the exacerbation of the detrimental effects associated with the progression of diabetes (summarized in Figure 1.11).

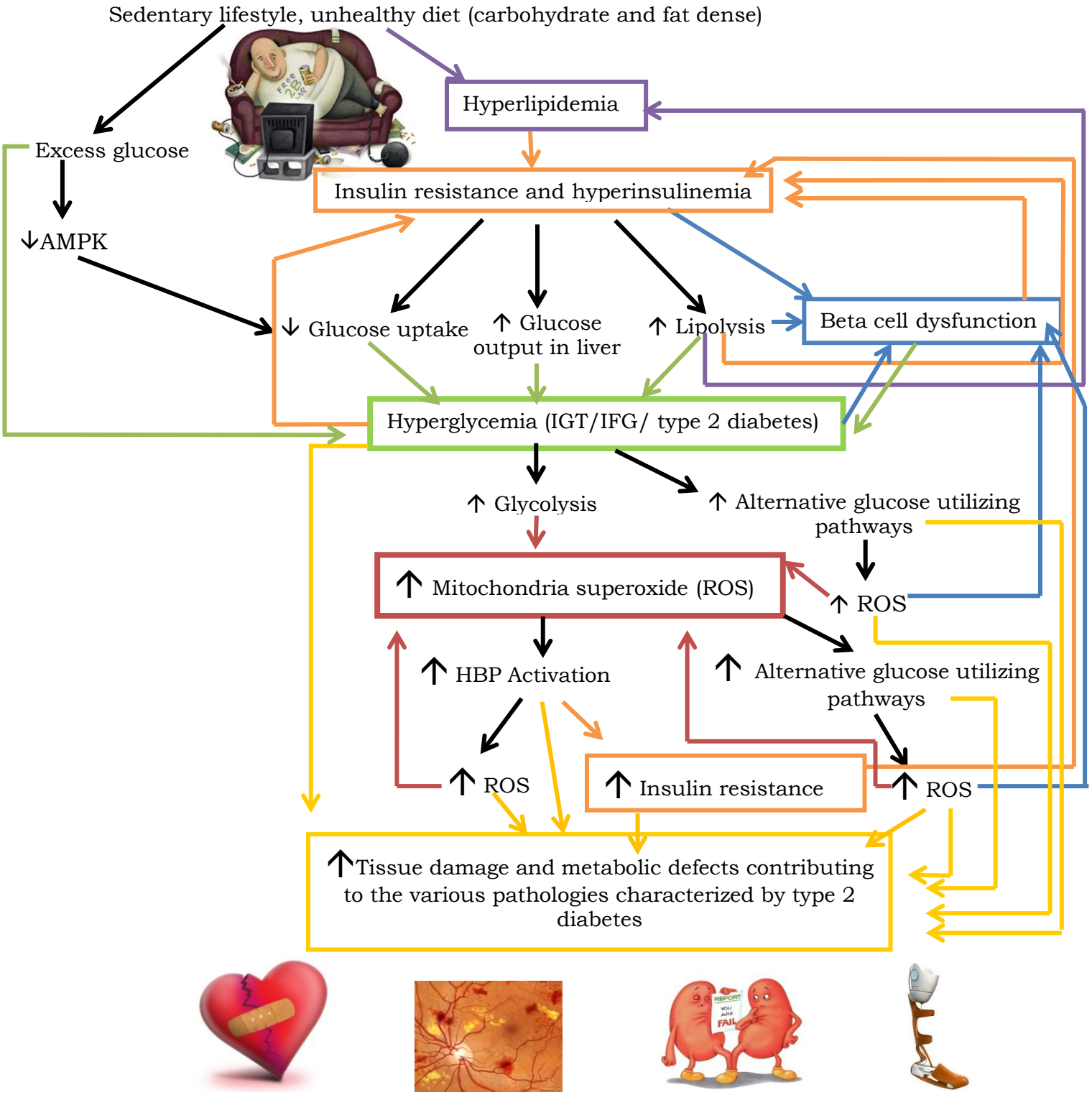


Figure 1.11 A summarized schematic representation of the pathophysiology and development of type 2 diabetes as reviewed in sections 1.1-1.6. This illustration emphasizes that, when put together, complex relationships exist between all the etiologies previously described. Additionally, it highlights the severity of various interlinking interactions as it yields a feed-forward system of progressive metabolic dysfunction.

Due to the strong association of the HBP with type 2 diabetes and insulin resistance and its ability to regulate cellular activities according to the nutritional environment of the cell, we investigated the utility of hyperglycemia-induced HBP up-regulation as a novel marker for insulin resistance/type 2 diabetes. Since this is the main focus of the current thesis, we will now proceed by focusing specifically on the HBP and its various cellular responses, beginning with a detailed overview of the pathway.

1.7 Overview of the HBP

The HBP is a cellular fuel-sensing pathway mediating an appropriate response in accordance with the nutritional status of the cell [189]. Moreover, the HBP is an integration of a variety of metabolic inputs (amino acid, glucose, fatty acid and nucleotide metabolism) (refer to Figure 1.12), enhancing its nutrient/glucose sensing abilities [190]. Under physiological conditions ~2-3% of total glucose is metabolized via the HBP. However, HBP flux may differ between diverse cell types [2].

The HBP shares its first two steps with glycolysis (conversion of glucose to glucose-6-phosphate (G-6-P), and the conversion of G-6-P to fructose-6-phosphate (F-6-P), at which point the HBP becomes independent (Refer to Figure 1.12) [188, 191]. GFAT is the rate-limiting HBP enzyme and is responsible for the catalytic conversion of F-6-P into glucosamine-6-P (GlcN-6-P) (Figure 1.12) [2]. A series of successive reactions result in GlcN-6-P being converted into uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc), the end-product of the HBP (Figure 1.12) [192]. A negative feedback loop exists at both the enzymatic production of GlcN-6-P and the HBP end-product (UDP-GlcNAc) [2]. Subsequently, UDP-GlcNAc serves as a substrate for the attachment of a single N-acetylglucosamine (*O*-GlcNAc) to serine

and threonine residues of certain nuclear and cytoplasmic proteins [188]. *O*-GlcNAc is dynamically attached and removed under tight regulation of two conserved enzymes, *O*-linked β-*N*-acetylglucosaminyl transferase (OGT) and β-*N*-acetylglucosaminidase (OGA), respectively [191, 193]. The interplay between these highly conserved enzymes is termed *O*-GlcNAc cycling, i.e. the catalyzed addition/removal of *O*-GlcNAc from proteins without any degradation of the polypeptide [192].

During either type 2 diabetes, or nutrient excess, the amount of F-6-P available to enter the HBP increases and as a consequence there is enhanced intracellular *O*-GlcNAcylation of target proteins [183, 194]. Moreover, glucosamine can be funnelled into the HBP (bypassing GFAT) thus further elevating *O*-GlcNAcylation (refer Figure 1.12). Surplus glucose flux into the HBP and the concomitant increase in *O*-GlcNAcylation are implicated in the development of insulin resistance and various cardiovascular pathologies commonly associated with type 2 diabetes (reviewed in [192-194]).

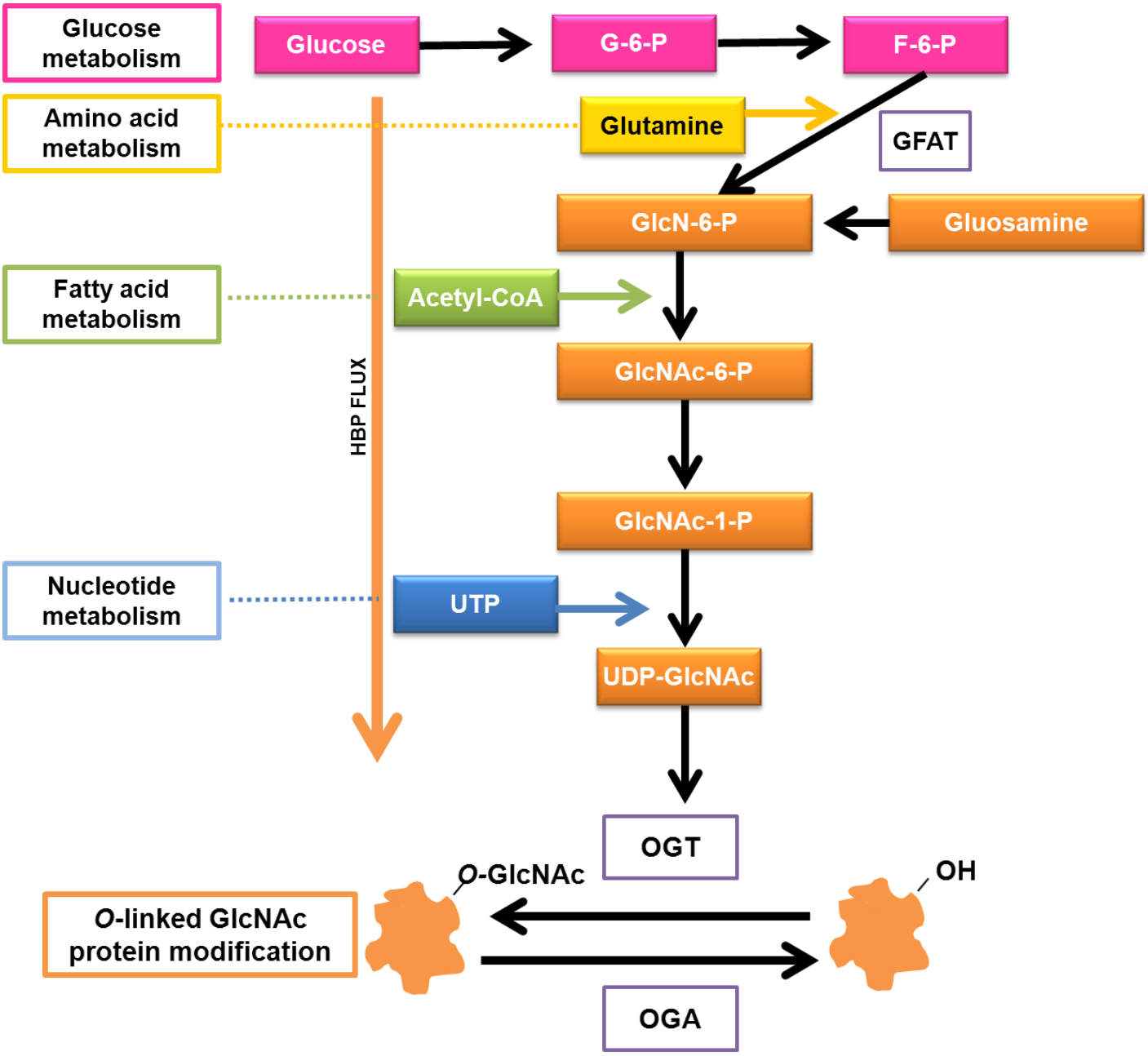


Figure 1.12 A schematic representation of the HBP (adapted from Hart, 2011). The HBP (represented by →and →), converts glucose into UDP-GlcNAc where OGT and OGT catalyse the attachment and removal of the *O*-GlcNAc moiety, respectively An advantage of the HBP is that it integrates additional metabolic inputs such as (→ amino acid metabolism), (→ fatty acid metabolism), and (→ nucleotide metabolism). G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; GFAT,glutamine:fructose-6-phosphate amidotransferase; GlcN-6-P, Glucosamine-6-phosphate; GlcNAc-6-P, GlcNAc-6-phosphate; GlcNac-1-P, GlcNAc-6-phosphate; UTP, uridine triphosphate; UDP-GlcNAc, uridine diphosphate GlcNAc, OGT, *O*-linked β-*N*-acetylglucosaminyl transferase ; OGA, β-*N*-acetylglucosaminidase.

1.8 Involvement of the HBP in insulin resistance and cardiac pathologies

The association between the HBP and the development of insulin resistance was first described by Marshall *et al.* (1991) in rat adipocytes [195]. These authors found that GFAT inhibition resulted in attenuation of the glucose-responsive desensitization of GLUTs [195]. Since then, several studies have supported the involvement of the HBP in the development of insulin resistance. For example, it was shown that GFAT overexpression in both skeletal muscle and adipose cells lead to the development of insulin resistance [196, 197]. Akimoto *et al.* (2007) found elevated *O*-GlcNAcylation and OGT levels with a concurrent deterioration of pancreatic insulin secretion in a rat model of type 2 diabetes [198]. Moreover, global *O*-GlcNAcylation in 3T3-L1 adipocytes lead to increased insulin intolerance [199] and the *in vivo* infusion of excess glucosamine has also been implicated in decreased sensitivity towards insulin in skeletal muscle [200]. A study conducted on rat adipocytes found that increased HBP flux decreases glucose uptake through the activation of PKC (see Figure 1.3) [201]. Furthermore, the overexpression of OGT has been implicated in the development of insulin resistance by altering GLUT4 transporters [202]. Additionally, a correlation has been found between a polymorphism in the *O*-GlcNAcase (OGA) gene and type 2 diabetes in Mexican-Americans [203], and *in vitro* studies show that OGA inhibition triggers insulin resistance [199]. *It is therefore apparent, from several lines of evidence, that O-GlcNAcylation and the enzymes that regulate this post-translational modification are associated with insulin resistance. However, exactly how does this happen?*

O-GlcNAcylation is a post-translational modification remarkably analogous to protein phosphorylation (reviewed in [204]). Due to their similarities, an extensive interplay exists between *O*-GlcNAcylation and phosphorylation and numerous

phosphorylation sites can indeed be *O*-GlcNAcylated [192]. The reciprocal occupancy between these two nucleocytoplasmic modifications plays an important role especially when *O*-GlcNAcylation is elevated. Here increased *O*-GlcNAcylation may influence/disrupt several vital phosphorylation reactions required for efficient signaling of various kinase cascades involved in intracellular function [192]. It should also be noted that the crosstalk is not strictly reciprocal and that certain target proteins can be concomitantly phosphorylated and *O*-GlcNAcylated [205]. Moreover, even adjacent *O*-GlcNAcylation sites may influence the phosphorylation of fundamental kinases [205].

The insulin signaling pathway is an example of an essential kinase-dependent cascade that is vulnerable to disruption by *O*-GlcNAcylation. Numerous studies found that increased *O*-GlcNAcylation attenuates the insulin-stimulated phosphorylation of IRS-1 and Akt and enhances the covalent attachment of *O*-GlcNAc to key regulatory components upstream of GLUT4 translocation. This includes IRS-1, PI3K, Akt and GLUT4 itself and eventually results in diminished glucose disposal [193, 205-207]. Furthermore, *O*-GlcNAcylation of proteins within the beta cells can affect insulin secretion [208] and *O*-GlcNAcylation of mitochondrial proteins may lead to mitochondrial dysfunction and insulin resistance [209].

Numerous studies confirm the direct involvement of *O*-GlcNAcylation in the development of insulin resistance, and these include different insulin-sensitive tissues such as muscle, liver and fat (reviewed in [192, 210]).

These studies therefore demonstrate that hyperglycemia-induced HBP activation and the subsequent increased *O*-GlcNAcylation has far reaching adverse effects in the onset of insulin intolerance. Moreover, numerous studies correlate increased *O*-GlcNAcylation with various vascular pathologies commonly associated with the development of type 2 diabetes [206, 211-213]. For example, increased *O*-GlcNAcylation contributes to cardiomyopathy through prolonged calcium transient decays and the down-regulation of the vital sarcoplasmic calcium ATPase (SERCA2a) [214]. Moreover, *O*-GlcNAcylation of key electron transport chain proteins can also result in cardiomyocyte mitochondrial dysfunction [215], while *O*-GlcNAc modified cardiac contractile proteins are proposed to contribute to diabetic-related malfunction in heart muscle [216]. Increased *O*-GlcNAcylation is also strongly associated with various cancers, aging, AIDS, and neurodegenerative diseases such as Alzheimers disease [182, 217].

We can thus infer from the above discussion that the role O-GlcNAcylation, OGT and OGA play in regulating insulin signaling and diabetes-associated cardiovascular diseases is robustly established. This correlation is vital in contributing to the potential diagnostic value of O-GlcNAc.

1.9 The *O*-GlcNAc modification

This novel protein-saccharide linkage was first described by Torres & Hart (1984) in lymphocytes [218]. It results in the dynamic attachment of a single sugar moiety (GlcNAc) to the hydroxyl groups of serine and threonine residues of nuclear and cytoplasmic proteins [219]. It is therefore essentially an alternative type of glycosylation [205]. Glycosylation is the enzymatic attachment of saccharides to site-specific molecules such as proteins and lipids [205]. However, *O*-GlcNAcylation

is an *O*-linked glycosylation owing to the addition of the carbohydrate to the hydroxyl oxygen of target proteins [205].

O-GlcNAcylation has emerged as an important post-translational modification with great interest due to its association with numerous critical biological functions, and also its implication as a pathogenic contributor to the progression of various diseases [219, 220]. A property unique to *O*-GlcNAcylation is that the saccharide linkage is not further extended or replaced by any other sugars as is the case for other types of glycosylation [205]. More significantly, this single monosaccharide modification has the ability to cycle several times at a time-scale similar to that of phosphorylation and can regulate cellular signaling dependent on substrate availability [221-223].

O-GlcNAc modification is ubiquitous and occurs in simple life forms e.g. protozoa up to higher mammals [220]. Thus far studies have shown that more than 1,500 proteins are *O*-GlcNAcylated and these include nearly every functional class [205]. Moreover, *O*-GlcNAcylation is essential and plays a significant role in many aspects of cellular metabolism such as transcription, translation, cytoskeletal assemblies, apoptosis, signaling and energy metabolism (reviewed in [188, 190, 205, 224]). However, the chronic elevation of *O*-GlcNAc in response to an overstimulation by nutrient stimuli leads to the alteration of both function and associations of target proteins, contributing to the pathology of diseases such as type 2 diabetes (reviewed in [190, 193, 225]). Interestingly, *O*-GlcNAcylation is regulated under the stringent control of only two enzymes, OGT and OGA, compared to the phosphorylation signaling network that is sustained by more than 500 kinases and phosphatases [188].

1.10 Regulation of the HBP

O-GlcNAcylation is robustly regulated by the concerted actions of two highly conserved enzymes i.e. OGT and OGA [190, 223, 225]. OGT is responsible for the catalytic attachment of *O*-GlcNAc to the hydroxyl residues of target proteins while OGA catalyzes hydrolysis of the glycosidic bond to remove *O*-GlcNAc from modified proteins (Figure 1.13) (reviewed in [188, 205, 226]).

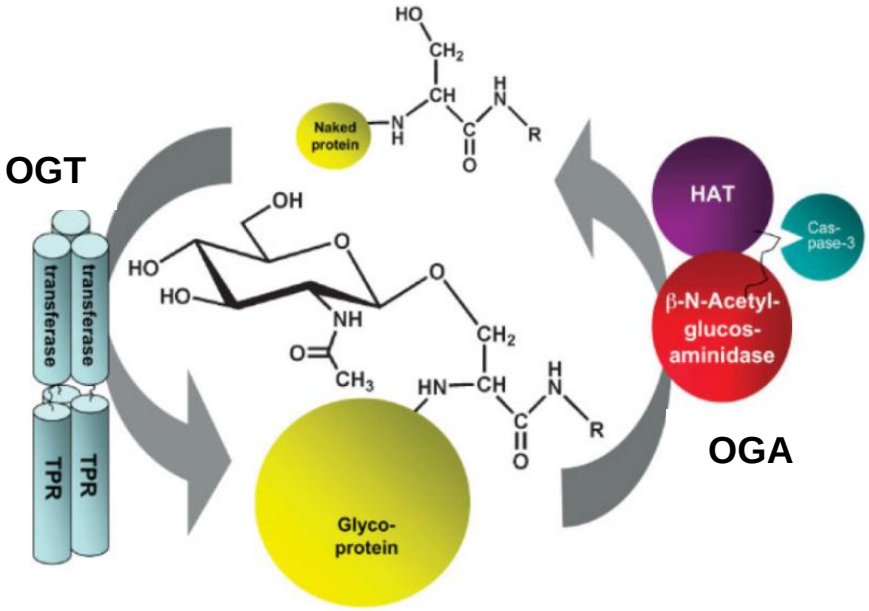


Figure 1.13 The dynamic attachment and cleavage of *O*-GlcNAc by OGT and OGA, respectively (Adapted from [225]). HAT, histone acetyl transferase; OGT, *O*-GlcNAc transferase; OGA, *O*-GlcNAcase; TPR, tetrotricopeptide repeats.

1.10.1 OGT

The human OGT gene is located at q13 on the X chromosome [225]. Originally, OGT was isolated from rat liver and it was evident that OGT existed as a heterotrimer consisting of two 110 kDa subunits and one 78 kDa subunit [227]. However, it has since been shown that the p110 subunit is capable of performing its catalytic activity independent of the p78 unit. Furthermore, the p78 splice variant is only expressed in the liver, kidney and muscle (discussed in [228, 229].

Therefore in other tissues that lack the p78 subunit it is expressed as a homotrimer comprising three 110 kDa subunits [227, 230]. The 110 kDa subunit is structured as two major domains bridged by a nuclear localization sequence [188, 225]. The N-terminal domain consists of several tetratricopeptide repeats (TPR) while the C-terminals encompass the catalytic domain (refer to Figure 1.13) (reviewed in [188, 225]). The exclusive substrate specificity of OGT is generally attributed to the TPR domains. However, the regulation of OGT activity is becoming increasingly complex and several aspects of its substrate specificity are still poorly understood [231]. Less is known regarding the structural properties of the C-terminus, although its catalytic power was confirmed by Lubus & Hanover (2000) [232]. OGT knock-out models reveal that OGT is essential for life due to its significant role in efficient growth and responsiveness towards extracellular stimuli [233].

1.10.2 OGA

The OGA gene is located at 10q on chromosome 24 [221]. OGA exists as a 106 kDa heterodimer complex comprising two subunits: a 54 kDa α and a 51 kDa β subunit [234]. The structural characteristics of OGA include a caspase-3 cleavage site that links *O*-GlcNAcase to an intrinsic histone acetyl transferase (HAT) domain [188] (See Figure 1.13). The single OGA gene in mammals is annotated as meningioma expressed antigen 5 (MGEA5) [226]. In humans the MGEA5 gene encodes nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase (NCOAT), a larger protein (130 kDa) within which the OGA enzyme resides [235].

1.11 The diagnostic utility of *O*-GlcNAc

In order to ensure the development of a practical and clinically useful diagnostic tool for diabetes, specific *O*-GlcNAc modified target proteins within the blood become the fundamental interest.

Erythrocytes are considered one of the simplest cells found within the bloodstream of the human body [87]. However, this deceivingly simple cell boasts a complex proteome consisting of intricate cellular processes [236]. Moreover, a recent study by Park *et al.* (2010) detected numerous *O*-GlcNAc protein sites in the erythrocyte proteome, and observed differential regulation of total *O*-GlcNAcylation between normal and diabetic samples [80].

As far as we are aware, Park *et al.* (2010) was the very first study to investigate the diagnostic utility of *O*-GlcNAc [80]. However no study has thus far investigated human leukocyte *O*-GlcNAcylation in a pre- and full-blown diabetic setting. By investigating leukocytes we hoped to gain additional insights and further advance the field of diabetes diagnosis with this novel premise. Since *O*-GlcNAc protein sites are found in both granulocytes and lymphocytes this broadens the scope of our investigation and potentially increases valuable outputs. Leukocyte proteins are therefore a suitable candidate for our initial and exploratory investigation into the diagnostic utility of *O*-GlcNAc in search of an eventual novel diagnostic marker for the earlier detection of type 2 diabetes.

1.12 Summary of research problem

Type 2 diabetes presents a formidable challenge due to its epidemic prevalence as well as its association with numerous adverse effects [237]. Individuals with diabetes are highly vulnerable to various CVD and several non-vascular conditions e.g. certain cancers, renal, digestive and infectious diseases [29]. It is estimated that 33% of individuals suffering from diabetes are unaware they are saddled with this disorder [84].

Although significant improvement has been made in the field of diabetes diagnosis, complexities and uncertainties still exist regarding the preferred diagnostic tests and the most efficient threshold values to be used during assessment [49]. Moreover, currently available tests for the diagnosis of diabetes have several shortcomings. **As delayed diagnosis leads to significant increases in morbidity and mortality, a novel screening method for the earlier and more efficient detection of type 2 diabetes would be a significant clinical advance.**

The diabetic phenotype is strongly associated with hyperlipidemia, hyperinsulinemia and hyperglycemia [90]. Hyperglycemia leads to the elevation of intracellular oxidative stress, primarily through the mitochondrial production of superoxide [146]. Hyperglycemia-induced oxidative stress results in the increased activation of five non-oxidative branches of glycolysis, including the HBP. Augmented flux through the HBP leads to the dynamic, post-translational attachment of *O*-GlcNAc moieties to serine and threonine residues of numerous nuclear and cytoplasmic proteins [192]. The HBP is an overflow pathway of glycolysis and a powerful consequence of this pathway is that *O*-GlcNAc modification is dependent on the availability of glucose, inaugurating the HBP as a

"fuel sensor" capable of regulating cellular activities according to the nutritional environment of the cell. This leads us to our hypothesis…

1.13 Hypothesis

As *O*-GlcNAc modification of target proteins is subject to intracellular glucose availability, the extent of *O*-GlcNAcylation may be a useful tool to assess glucose metabolism of individuals with both subtle and overt glucose dysregulation [27, 188, 192]. Moreover, chronically elevated HBP flux is maladaptive and is strongly linked to the onset of insulin resistance and various vascular pathologies associated with type 2 diabetes [188]. *O*-GlcNAc moieties are dynamically attached and removed from target proteins under the robust control and combined efforts of OGT and OGA, respectively [26, 238]. Since *O*-GlcNAc protein sites are found in granulocytes and lymphocytes, we hypothesize **that increased *O*-GlcNAcylation of leukocyte proteins in the pre-diabetic and diabetic milieu could potentially represent a novel diagnostic marker for the earlier detection of type 2 diabetes.**

1.14 Aims and objectives

- To recruit subjects and obtain blood samples representing a range of blood glucose levels, from normal to pre-diabetic and diabetic.

- To characterize individuals according to the ADA and WHO's classifications, with both FPG and HbA1c criteria

- Determine the level of total protein *O*-GlcNAcylation in the leukocytes of different subjects investigated in this study

- Investigate the expression of the HBP's regulatory enzymes (OGA and OGT) in the leukocytes of all study subjects

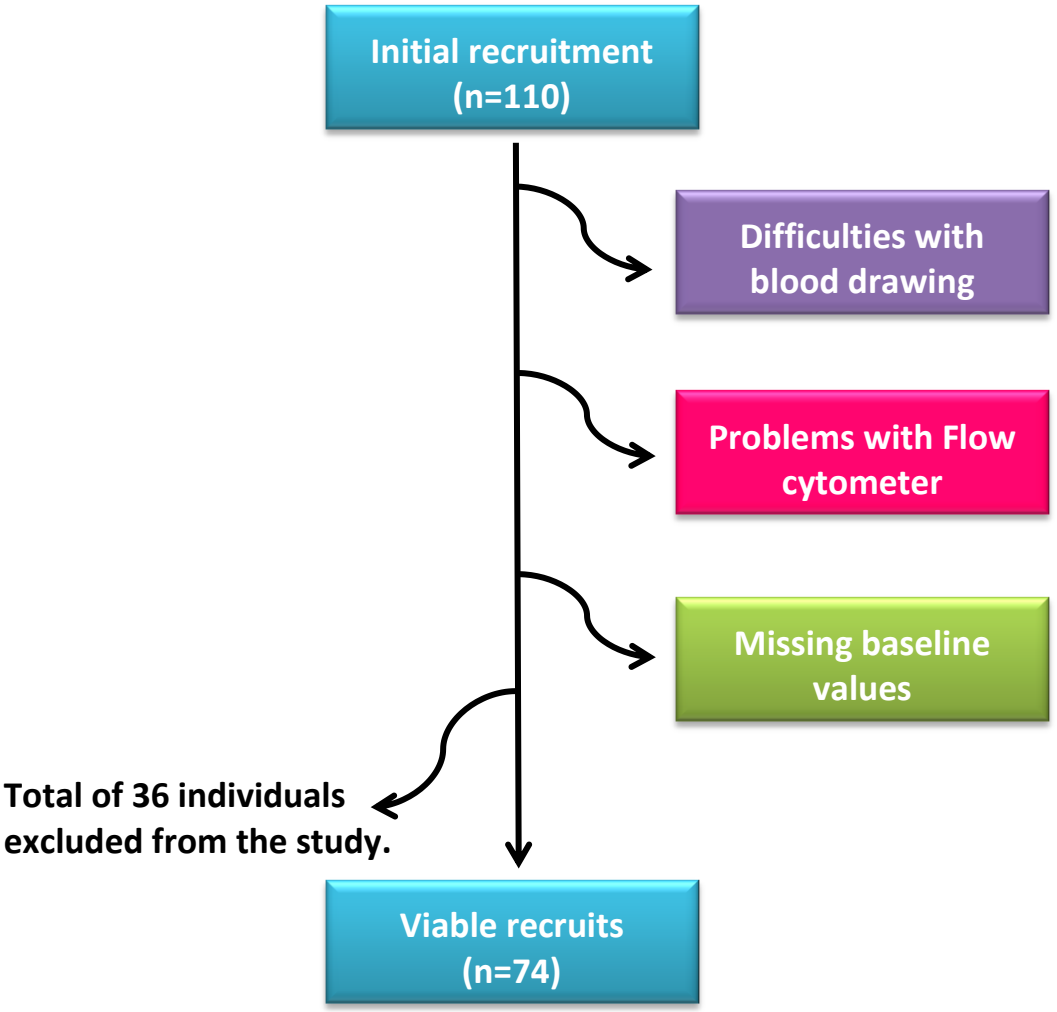
- Explore HBP flux in the different leukocyte sub-populations (granulocytes versus lymphocytes), independently from total leukocyte samples

- To execute a simple, concise, exploratory phase of a novel project with the primary aim to determine if there is impetus for further investigation with the eventual aim to offer diagnostic utility

- To further develop the field of diabetes diagnosis by providing an overview of the current status of the field, providing clarity regarding to the on-going flaws, and likewise highlighting various discrepancies and the ensuing effects of such inconsistencies. With this we hope to underscore the importance of efficient diagnosis and to make a significant contribution to the success of future endeavors focusing on the advancement of diagnosing diabetes.

Materials and Methods

2.1 Subject recruitment



Participants (n=74) were recruited from two closely located urban areas, i.e. Bellville (n=59) and nearby Stellenbosch (n=15) (Western Cape, South Africa). Baseline characteristics of recruited subjects are summarized in Table 2.1. All enlisted participants were informed about the clinical study (verbally or via e-mail) and provided with a written consent form explaining all the aims and procedures. Study volunteers could withdraw from the study at any point without any explanation required. This study was approved by the Committee for Human

Research at Stellenbosch University and was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research in South Africa (Reference numbers: N09/03/090 and N09/06/168).

	Normal	Pre-diabetes	Diabetes
Sample size	27	16	31
Age (years)*	53.4 ± 2.4	57.6 ± 4.0	61.7 ± 1.6
Gender (M/F)	6/21	6/10	11/20
Fasting glucose (mmol/L)*	4.9 ± 0.07	6.1 ± 0.09	11.4 ± 0.75
HbA1c (%)*	6.1 ± 0.2	6.2 ± 0.09	9.1 ± 0.4

Table 2.1 Baseline characteristics of recruited individuals.

*Values expressed as mean ± SEM

2.2 Characterization of subjects

Due to the ongoing debate and lack of consensus regarding both the preferred screening method for the detection of diabetes as well as organization-specific characterization criteria (discussed in section 1.3.2), we categorized subjects according to both ADA and WHO classifications (FPG and HbA1c criteria). The WHO's criteria do not make a distinction between normal and pre-diabetic individuals when interpreting HbA1c levels. We thus used the ADA's recommendations for HbA1c while for FPG levels, two sets of criteria (WHO, ADA) were implemented. Each individual was therefore characterized into one of three groups (normal, pre-diabetic, diabetic) according to three different criteria i.e. a) ADA FPG, b) WHO FPG, and c) HbA1c (ADA) (Refer Figure 2.1).

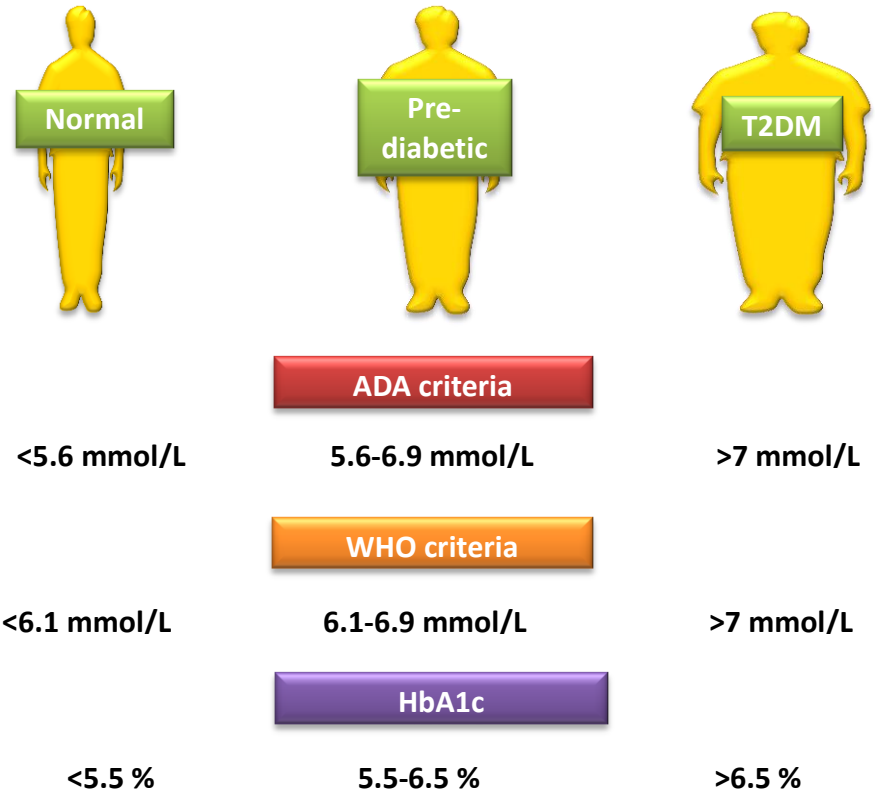


Figure 2.1 Subject characterization according to fasting plasma glucose (ADA, WHO) and HbA1c levels.

2.3 Sample collection

Whole blood samples were collected from volunteers under fasting conditions by venipuncture. Here blood tubes were collected into four sodium fluoride/potassium oxalate vacuette and one ethylenediaminetetraacetic acid (EDTA) tubes for each volunteer (Greiner Bio-one, Kremsmünster, Austria). Clinical data collected include measurements for FPG, and HbA1c levels (two blood tubes sent to Pathcare labs Pty Ltd. [Stellenbosch, South Africa]). For molecular studies (flow cytometry, immunofluorescence microscopy, Western blotting), the three remaining blood tubes were immediately centrifuged at 1000 x g at 4°C for ten minutes (Beckman Coulter Allegra X-22R centrifuge, Sigma-Aldrich, Steinheim, Germany), and separated into plasma, leukocytes and erythrocytes.

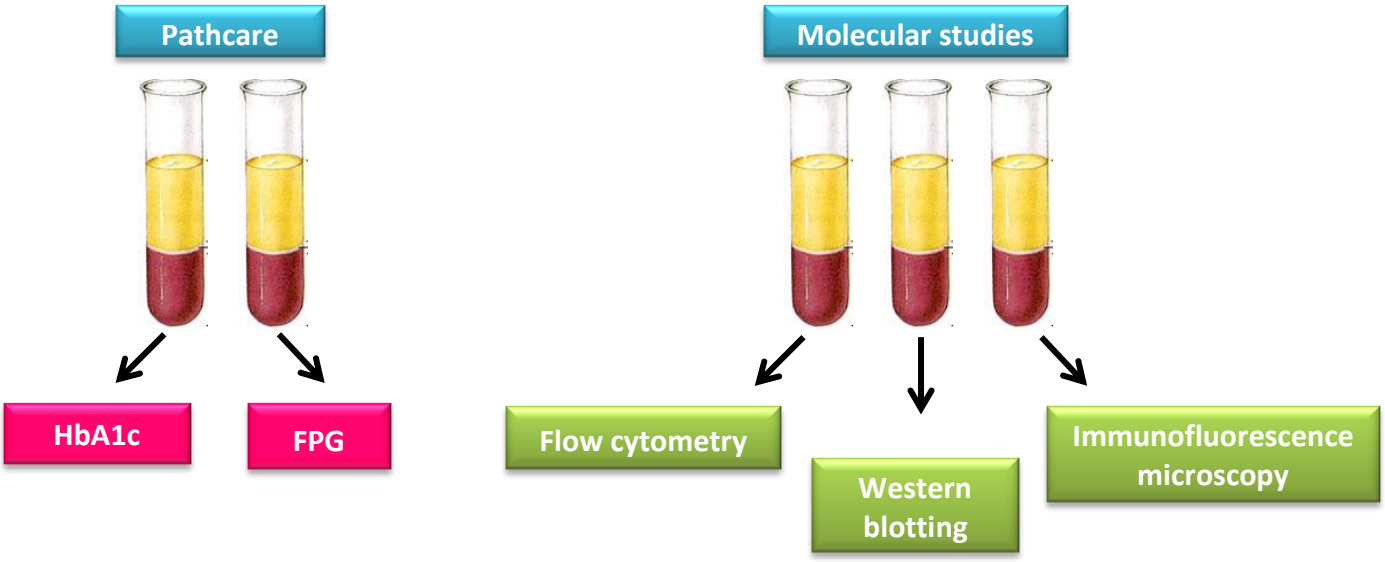


Figure 2.2 Collection of blood samples for clinical data and for molecular analysis.

2.4 Leukocyte isolation

Initially, two alternative methods of leukocyte isolation were tested and compared. Isolation using Histopaque®-1077 and Histopaque®-1119 (Sigma-Aldrich, St. Louis, MO), and manual isolation with a pipette both yielded a pure leukocyte sample (tested by flow cytometry). The Histopaque method, however, was time-consuming and more costly. We therefore opted for isolating leukocytes manually since it also offered increased efficiency. Details of both isolation techniques are described in sections 2.4.1 and 2.4.2 of this thesis.

2.4.1 Histopaque

3 ml of Histopaque®-1119 was added to a sterile 15 ml plastic "Falcon" tube (BD Biosciences, San Jose, CA), onto which another 3 ml of Histopaque®-1077 was carefully layered. Thereafter 4 ml of whole blood was cautiously added to the Histopaque double layer. After centrifugation at 700 x g for 30 minutes at room temperature leukocyte samples were obtained by aspirating cells from layer A and layer B (Figure 2.3).

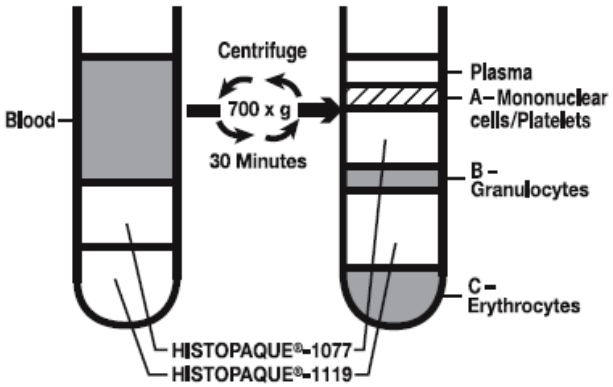


Figure 2.3 Isolation of leukocytes using Histopaque®-1077 and Histopaque®-1119.

2.4.2 Manual isolation

After centrifugation (1000 x g at 4°C for 10 min), the plasma from each blood tube was discarded and the white buffy layer carefully isolated (using a pipette) from the layer of erythrocytes. To ensure a purified leukocyte sample, residual erythrocytes (still found within buffy coat) were lysed by adding 1x BD FACS lysing solution (BD Biosciences, San Jose, CA), which lyses erythrocytes but not leukocytes, followed by centrifugation for 5 min at 500 x g at room temperature. The pellet was thereafter washed twice with cold PBS and split into three fractions, i.e. leukocytes from one tube were rapidly stored at -80°C for Western blotting, while the

leukocytes from the remaining two tubes were promptly processed for flow cytometry and immunofluorescence microscopy.

2.5 Investigation of HBP flux

Here we determined the extent of leukocyte *O*-GlcNAcylation and the expression of OGT and OGA, key HBP regulatory enzymes. Leukocyte total *O*-GlcNAcylation was evaluated by using three alternative techniques, i.e. flow cytometry, immunofluorescence microscopy and Western blotting.

2.5.1 Flow cytometry

2.5.1.1 *O*-GlcNAcylation

Leukocytes (suspended in PBS) were permeabilized by addition of 500 µl of 10x BD FACS permeabilizing solution (BD Biosciences, San Jose, CA) for 5-10 min at room temperature. Cells were thereafter incubated on ice for 20-30 min with a 1:500 dilution of *O*-GlcNAc primary antibody (CTD 110.6, Santa Cruz Biotechnology, Santa Cruz, CA). Samples were subsequently centrifuged 500 x g at 15°C for five min, followed by 1 x PBS washing. After gentle resuspension, the sample was incubated for ten min on ice with a 1:200 dilution of goat anti-mouse allophycocyanin (APC) secondary antibody (Invitrogen, Carlsbad, CA). Cell suspensions were thereafter centrifuged at 500 x g for five min and the pellets resuspended in 500 µl PBS before analysis using a FACS Aria flow cytometer (BD Biosciences, San Jose, CA). We typically analyzed 10,000 cells per experiment and the signal quantified by determining the geometric mean of fluorescence for each specific cell population (protocol modified from [239]).

2.5.1.2 OGT

The determination of OGT expression with flow cytometry was completed as described in section 2.5.1.1. However, we instead employed the following primary and secondary antibodies:

Primary antibody: *O*-linked N-acetylglucosamine transferase (OGT) (Abcam, Cambridge, MA);

Secondary antibody: Anti-rabbit FITC secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

2.5.1.3 OGA

We determined OGA expression by flow cytometry as described in section 2.5.1.1. However, we instead employed the following primary and secondary antibodies:

Primary antibody: NCOAT L-14 (nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase) (Santa Cruz Biotechnology, Santa Cruz, CA);

Secondary antibody: Anti-goat FITC secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

2.5.2 Immunofluorescence microscopy

2.5.2.1 *O*-GlcNAcylation

The leukocyte fraction was fixed with 6% formaldehyde/PBS for 30 min and resuspended in PBS. We subsequently seeded 100 µl of cell suspension per well of 8-well Nunc™ cover glass chambers (Nalge Nunc, Rochester, NY), followed by 24 hours incubation at 37°C. Leukocytes were washed three times with 100 µl cold PBS before being permeabilized with methanol (-20°C) for 2 min. After the

chambers were left to air dry for 20 min, non-specific sites were blocked with 5% donkey serum (in PBS) for 30 min at room temperature. The cells were then incubated for 90 min at 37°C in 1:250 dilution of *O*-GlcNAc primary antibody (CTD 110.6, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 5% donkey serum. The chambers were thoroughly washed (4-5x) with 100 µl cold PBS before incubation with 1:250 dilution of Texas Red anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) for 45 min in the dark at room temperature. Thereafter, the chambers were washed with cold PBS (4-5x) before adding 50 µl of a 1:200 dilution of Hoechst in PBS (Sigma, St. Louis, MO) for 10 min. Cells were subsequently viewed using an Olympus Cellˆ R fluorescence 1 X 81 inverted microscope (Olympus Biosystems, Hamburg, Germany) and images were acquired with an F-view II camera with 60x magnification (Olympus Biosystems, Hamburg, Germany) (protocol modified from [240]). A Xenon-Arc burner (Olympus Biosystems, Germany) was used as a light source, and images were excited with the 572 nm (TxRed), 492 nm (FITC), and 360 nm (DAPI) excitation filters. Emission was collected using a UBG triple-bandpass emission filter cube. The Cell^R imaging software (Olympus Biosystems, Germany) was used for background subtraction, image processing and signal quantification.

2.5.2.2 OGT

OGT expression was evaluated by immunofluorescence microscopy as described in section 2.5.2.1. However, we instead employed the following primary and secondary antibodies:

Primary antibody: *O*-linked N-acetylglucosamine transferase (OGT) (Abcam, Cambridge, MA);

Secondary antibody: Anti-rabbit FITC secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

2.5.2.3 OGA

The determination of OGA expression with immunofluorescence microscopy was completed as described in section 2.5.2.1, but with modifications i.e. with the substitution of the following primary and secondary antibodies:

Primary antibody: NCOAT L-14 (nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase) (Santa Cruz Biotechnology, Santa Cruz, CA);

Secondary antibody: Anti-goat FITC secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

2.5.3 Western blotting

2.5.3.1 Protein extraction and quantification

Frozen leukocyte samples were thawed and sonicated for 10 sec in order to disrupt the cell membranes and expose the contents of the cell (refer to Appendix for complete lysate preparation protocol). The samples were then centrifuged at 4°C at 2500 x g for 10 min. We quantified protein using the Bradford protein determination method [241](see Appendix for complete protocol).

2.5.3.2 Sample preparation

Aliquots diluted in sample buffer and containing 20 µg of protein were prepared for all samples. The sample buffer used contained 850 µl 3x sample buffer and 150 µl mercaptoethanol (3x sample buffer: 0.5 M Tris, pH 6.8, 0.2 ml 0.5% bromophenol blue, 2.5 ml glycerol, 10% SDS and distilled water). A complete protocol for sample preparation is included in the Appendix.

2.5.3.3 SDS PAGE and Western blot analysis

Samples were boiled for 5 min (Refer to Appendix), loaded onto Bio-Rad Mini-Protean® TGX™ –Pre-cast gels (Bio-RAD Laboratories, CA), and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. 4 µl of protein marker ladder (Bio-RAD PlusProtein™ Dual Color Standards, USA) was loaded in the first lane. Samples were run for 25 min at 250 V (constant) and 400 mA. Thereafter, proteins were transferred onto a 0.2 µM PVDF using the Bio-Rad Trans-Blot® Turbo™ Transfer system V1.02 (Bio-RAD Laboratories, CA). Proteins were transferred for 12 min at 15 V and 0.5 A. After transfer, membranes were washed three times (duration of wash: 5 min) in Tris Buffered Saline-Tween-20 (TBS-T) before blocking against non-specific binding in 1% (w/v) bovine serum albumin (BSA) in 0.1% TBS-T for 15 min at room temperature. After blocking, the membranes were washed three times in TBS-T and incubated overnight at 4°C with 1:1000 of the appropriate primary antibody (see Table 2.2). The following day the membranes were thoroughly washed in TBS-T and incubated in 1:4000 of the appropriate secondary antibody (See table 2.2) for 60 min at room temperature. The membranes were again washed with TBS-T (3 x 5 min) before evenly adding 1 ml of KPL LumiGLO (KPL Inc, MD) over each membrane for one min. The membrane was developed using the Bio-Rad Chemidoc™ MP Imaging system (Bio-RAD Laboratories, CA). Bio-Rad Image Lab™ Software version 4.0 (Bio-RAD Laboratories, CA) was used to quantify Western blots through volume analysis.

	Primary antibody	Secondary antibody
***O*-GlcNAc**	*O*-GlcNAc (CTD 110.6, Santa Cruz Biotechnology, Santa Cruz, CA*)	Goat anti-mouse antibody (31440, Thermo Scientic, Rockford, IL)
OGT	OGT (AL28 *)	Goat anti-rabbit HRP (611 1302, Rockland Immunochemicals, PA)
OGA	NCOAT L-14 (Santa Cruz Biotechnology, Santa Cruz, CA)	
β-Actin	β-actin (4976S, Cell Signalling Technology, Boston, MA)	Goat anti-rabbit HRP (611 1302, Rockland Immunochemicals, PA)

Table 2.2 Primary and secondary antibodies used for Western blotting analysis. *We wish to thank the Gerald Hart laboratory, Johns Hopkins and the NHLBI P01HL107153 Core C4 for providing the OGT (AL28) and CTD 110.6 antibodies.

2.6 Differentiation of leukocyte subtypes

We employed flow cytometry to distinguish between granulocytes and lymphocytes and used a gating strategy with both scatter and fluorescence parameters. Since the FACS Aria flow cytometer measures linear forward light scatter (FSC) and linear 90° light scatter (SSC), it allowed separation of leukocytes according to size and cell granularity, respectively. A fluorescent-conjugated marker present in all leukocytes (CD45) (Abcam, Cambridge, MA) was employed to define leukocyte sub-groups. We conjugated CD45 to either a FITC or APC fluorescent probe, followed by two-color immunofluorescence. Here leukocyte samples were stained with CD45-FITC or CD45-APC ± either of *O*-GlcNAc-APC, OGA-FITC or OGT-FITC. The accurate identification of granulocytes and lymphocytes was completed by using a SSC/CD45 scatter plot (granulocytes: high SSC, low CD45 signal; lymphocytes: low SSC, high CD45 signal). The relative amounts of *O*-GlcNAc, OGA and OGT could also be determined within the different leukocyte populations. We typically analyzed 10, 000 cells per experiment and the signal quantified by determining the

geometric mean of fluorescence for each specific cell population (protocol modified from [242]).

2.7 Determining the effect of insulin on HBP flux

Due to an interesting finding in our study (refer to Results section 3.14.2), we decided to investigate (cell culture model) whether insulin could have an effect on the extent of O-GlcNAcylation. To our knowledge, no literature has elucidated this before. We employed Western blotting and immunofluorescence microscopy for this analysis.

2.7.1 Cell culture

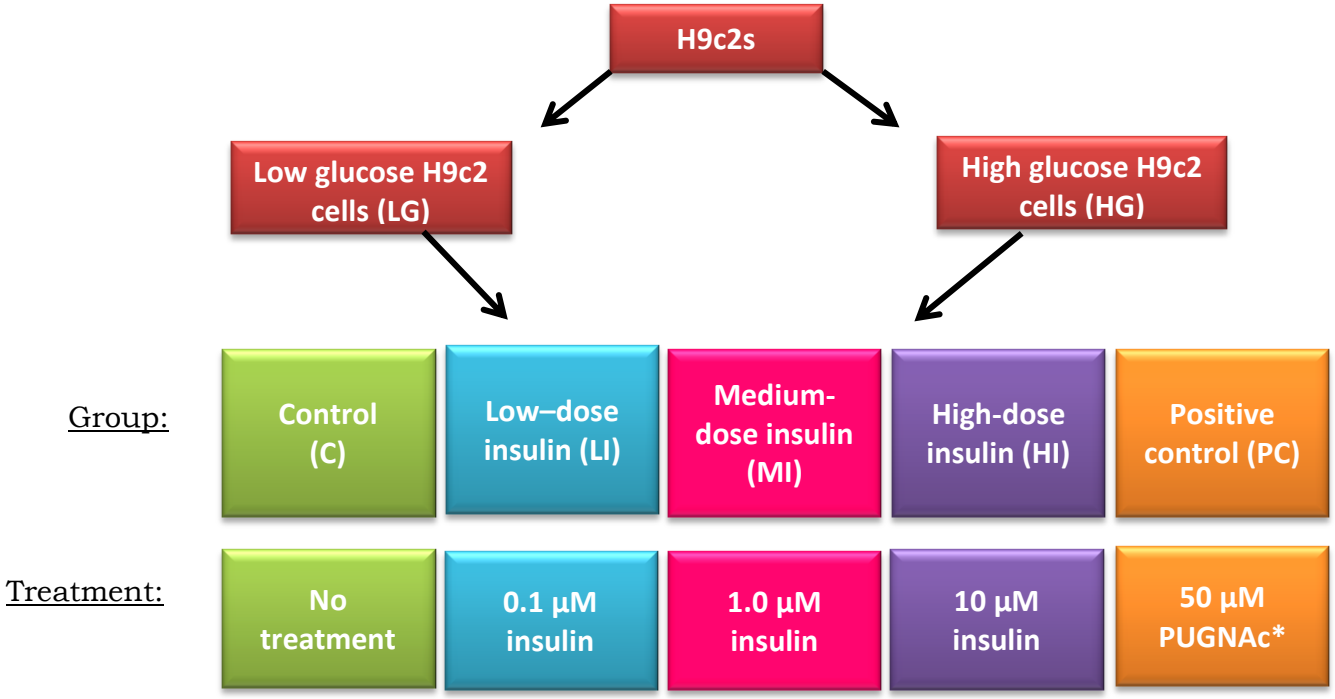
Experiments were performed using an H9c2 rat cardiomyoblast cell line (Sigma-Aldrich, Steinheim, Germany), a precursor to cardiomyocytes. Cells were cultured in T75 culture flasks (75 cm^2 flasks, Greiner Bio One, Germany) at 37˚C (5% CO_2, 95% air) in Dulbecco Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco Invitrogen, Carlsbad, CA) and 1% Penicillin-Streptomycin solution (PenStrep) (Gibco Invitrogen, Carlsbad, CA). Two groups of H9c2s were cultured, i.e. a high glucose group in 25 mM DMEM (simulating hyperglycemia), and a low glucose group in 5.5 mM DMEM (simulating normoglycemia).

Cells proliferated in T75 flasks until they reached 80% confluency (between three and four days), after which they were split. The latter was accomplished by washing the cell monolayer with warm phosphate buffered saline (PBS) followed by incubation with 4 ml trypsin (Sigma Chemical Co., St Louis, MO) at 37°C for four min. Trypsinized cells were then neutralized with DMEM, counted with a hemocytometer and centrifuged at 500 x g for 3 min before seeding into appropriate

treatment T25 flasks (Western blotting) or cover glass chambers (immunofluorescence microscopy).

2.7.2 Experimental groups

High glucose cells and low glucose cells were each split into five experimental groups, resulting in a total of ten groups (Figure 2.4). For Western blotting, ten T25 flasks were seeded (one flask per group) as indicated in Figure 2.4. For immunohistochemistry, two 8-well Nunc™ cover glass chambers (Nalge Nunc, Rochester, NY) were used. Low glucose and high glucose cells were seeded in separate chambers, and in each chamber the insulin-treated groups were seeded in duplicate. Each chamber contained a control group and a dye control (DC) group. A dye control group (no primary antibody) was added when performing immunofluorescence staining to ensure antibody specificity.



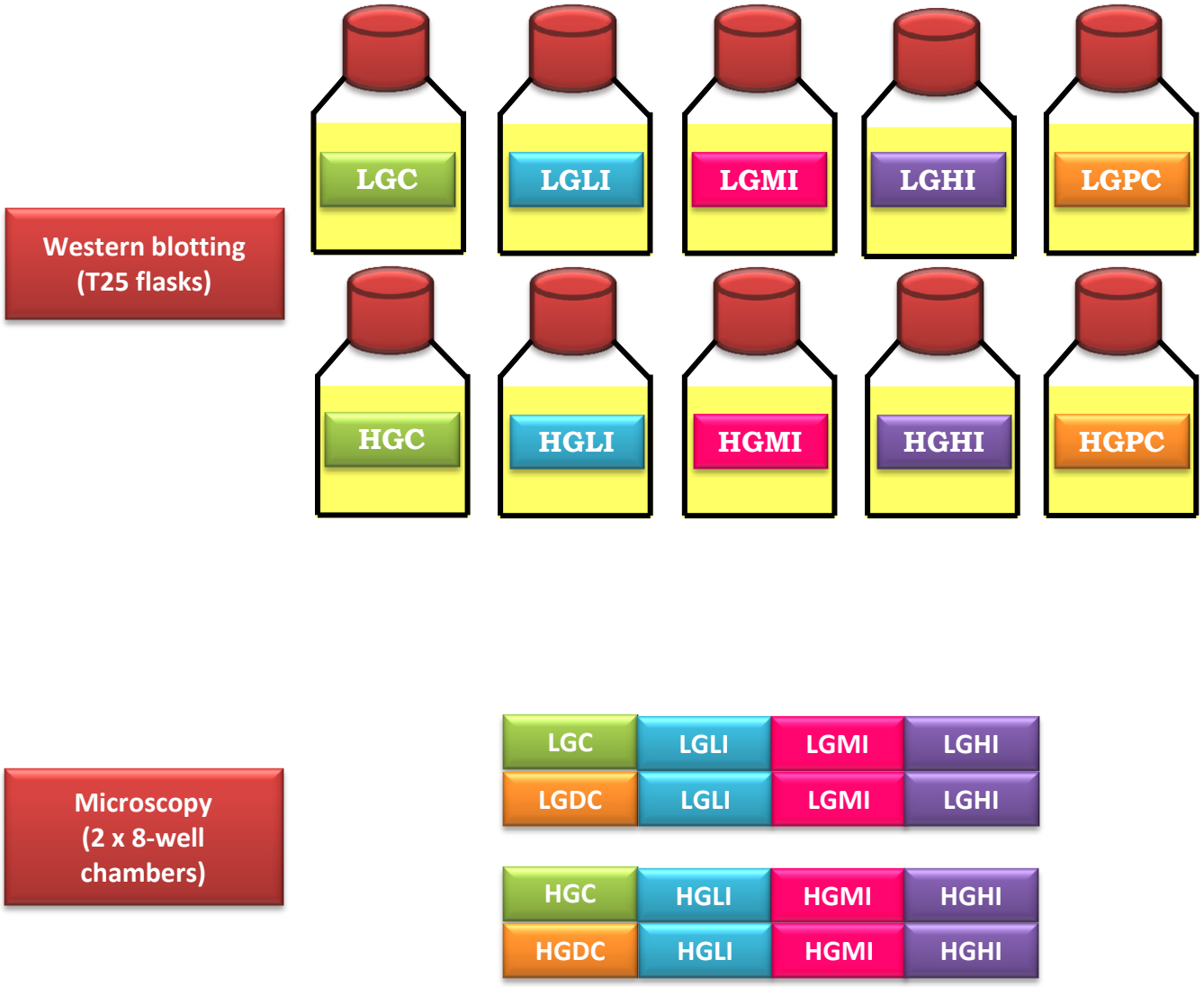


Figure 2.4 Schematic representation of experimental treatment groups used for Western blotting and immunofluorescence microscopy. LGC, low glucose control; LGLI, low glucose low-dose insulin; LGMI, low glucose medium-dose insulin; LGHI, low glucose high-dose insulin; LGPC, low glucose positive control; LGDC, low glucose dye control; HGC, high glucose control; HGLI, high glucose low-dose insulin; HGMI, high glucose medium-dose insulin; HGHI, high glucose high-dose insulin; HGPC, high glucose positive control; HGDC, high glucose dye control.

*PUGNAc (O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N phenylcarbamate) is an inhibitor of OGA (the removal of *O*-GlcNAc moieties), and is therefore a valuable positive control in Western blotting.

2.7.3 Western blotting

2.7.3.1 Preparation

On day 1, H9c2s were trypsinized from T75 flasks, and 500, 000 cells were added to ten T25 flasks (see Figure 2.4). On day 3, the medium was refreshed, and on day 4 the experiment took place. All medium in the flasks were substituted with serum-free medium for three hours prior to treatment. A period of serum starvation is necessary before insulin treatment in order to stimulate the translocation of GLUT4 to the sarcolemma after insulin treatment. One hour prior to insulin treatment, the positive control groups were treated with PUGNAc (CarboGenLabs, Aarau, Switzerland). Thereafter, the appropriate doses of insulin (Figure 2.4) were added to the insulin treatment flasks for 20 min before harvesting the cells. Serum-free DMEM was removed from all flasks and cells were washed with cold PBS and immediately put on ice. In order to extract cellular proteins, 1 ml of modified radioimmunoprecipitaion (RIPA) buffer was added to each flask for 10 min (RIPA: pH 7.4, containing: Tris–HCl 2.5 mM, EDTA 1 mM, NaF 50 mM, NaPPi 50 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride (PMSF) 0.1 mM, benzamidine 1 mM, 4 mg/ml SBTI, 10 mg/ml leupeptin, 1% NP40, 0.1% SDS and 0.5% Na deoxycholate) (refer to Appendix). Adhering cells were then harvested from the surface of the flask using a cell scraper (See Appendix for complete cell harvesting protocol), and lysates were stored at -80°C for future analysis by Western blotting. This experiment was repeated three times for statistical power.

2.7.3.1 Western blot analysis

Identical to protocol described in section 2.5.3.

2.7.4 Immunofluorescence microscopy

2.7.4.1 Preparation

On day 1, H9c2s were trypsinized from T75 flasks, and 150, 000 cells were added to each well of the 8-well Nunc™ cover glass chambers (Nalge Nunc, Rochester, NY) (see figure 2.4). On day 3, the medium was refreshed, and on day 4 the experiment took place. All medium in the wells were substituted with serum-free medium for three hours prior to treatment. A period of serum starvation is necessary before insulin treatment in order to stimulate the translocation of GLUT4 to the sarcolemma after insulin treatment. After three hours, the appropriate doses of insulin were added to the insulin treatment wells for 20 min before harvesting the cells. Serum-free DMEM was removed from all wells and cells were washed with cold PBS and immediately fixed with 6% formaldehyde/PBS for 30 min. The protocol for immunofluorescence *O*-GlcNAc staining was executed directly after the 30 min fixation period.

2.7.4.1 Immunofluorescence staining

Identical to protocol described in section 2.5.2.1, however, for the dye control, no *O*-GlcNAc primary antibody was added.

2.8 Statistical analysis

All values are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to determine differences between normal, pre-diabetic and diabetic subjects, followed by two Bonferroni *post hoc* tests. A Bonferroni test that compared all pairs of columns (denoted with *) and a

Bonferroni test comparing selected pairs of columns (denoted with #) was performed with each analysis. All statistical analyses were performed using Graphpad Prism version 5.01 (Graph pad Software Inc, USA). P values < 0.05 were accepted as significant.

Results

3.1 Leukocyte flow cytometric scatter properties and differential CD45 intensity define two major leukocyte populations

We employed flow cytometry to measure a) light scatter properties and b) CD45 fluorescent intensity in the total leukocyte sample. Firstly, FSC and SSC properties separated the total white blood cell population according to physical characteristics (size and cell granularity, respectively) [243]. The diverse physical properties of leukocyte sub-populations allowed two main sub-groups to be distinguished from one another and from cellular contaminants, namely lymphocytes (green) and granulocytes (blue) (Figure 3.1A).

To confirm these findings, we also measured the mean CD45 fluorescence intensity. CD45 is a marker found on all leukocytes, but with varying intensity within different leukocyte sub-groups [243]. Additionally, since CD45 is absent from erythrocytes, it is an ideal marker for the accurate differentiation between white blood cell (WBC) sub-types [244]. The leukocyte sample was plotted on a SSC/CD45 axis and again the two sub-populations were gated. Lymphocytes (green) exhibited a low SSC and a high CD45 signal intensity versus a low CD45 fluorescence and high SSC characteristic of granulocytes (blue) (Figure 3.1B). These data were robustly supported by a SSC/FSC scatter plot demonstrating a stronger CD45 signal (red) in lymphocytes (green) compared to granulocytes (blue) (Figure 3.1C). Thus our flow cytometric protocol allows us to distinguish two leukocyte sub-populations with great accuracy.

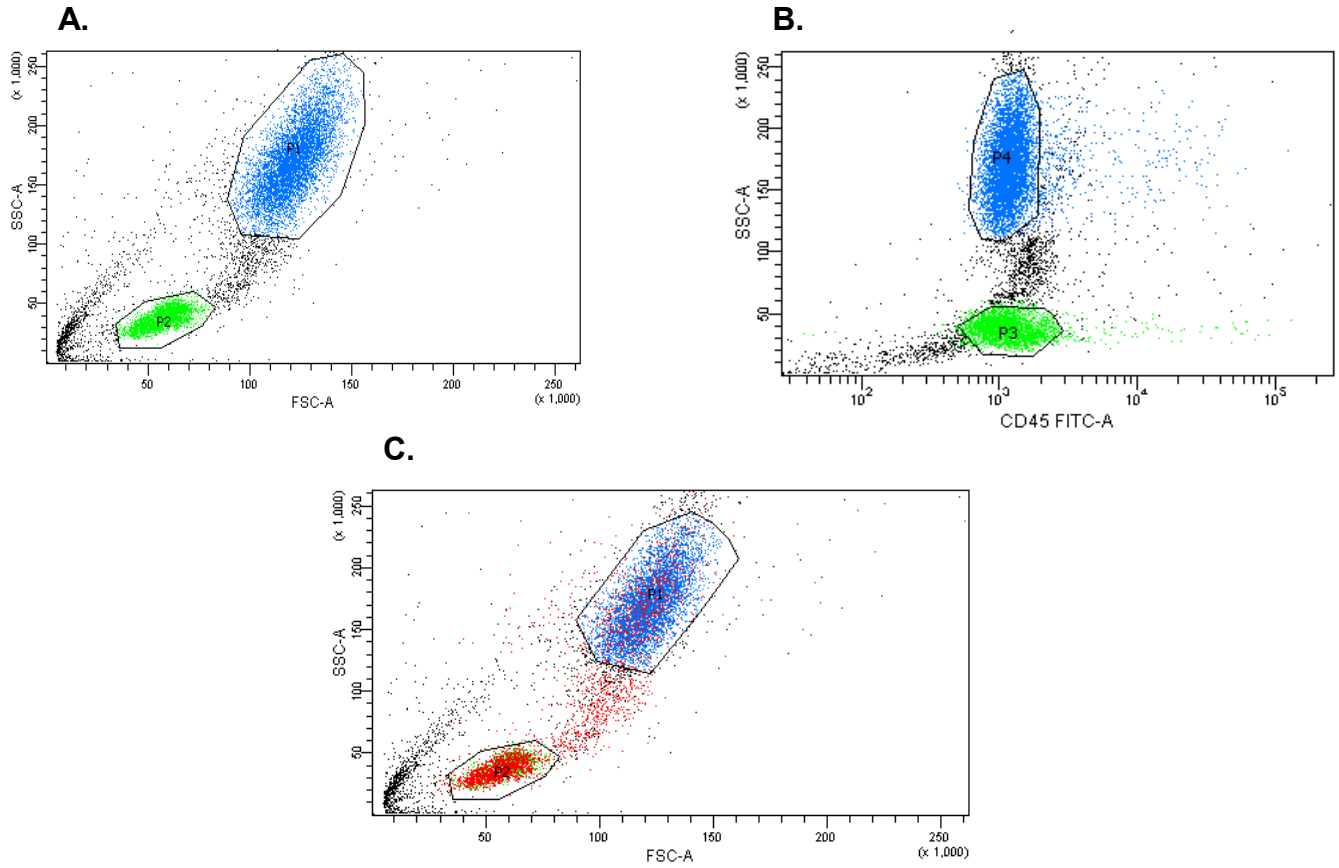
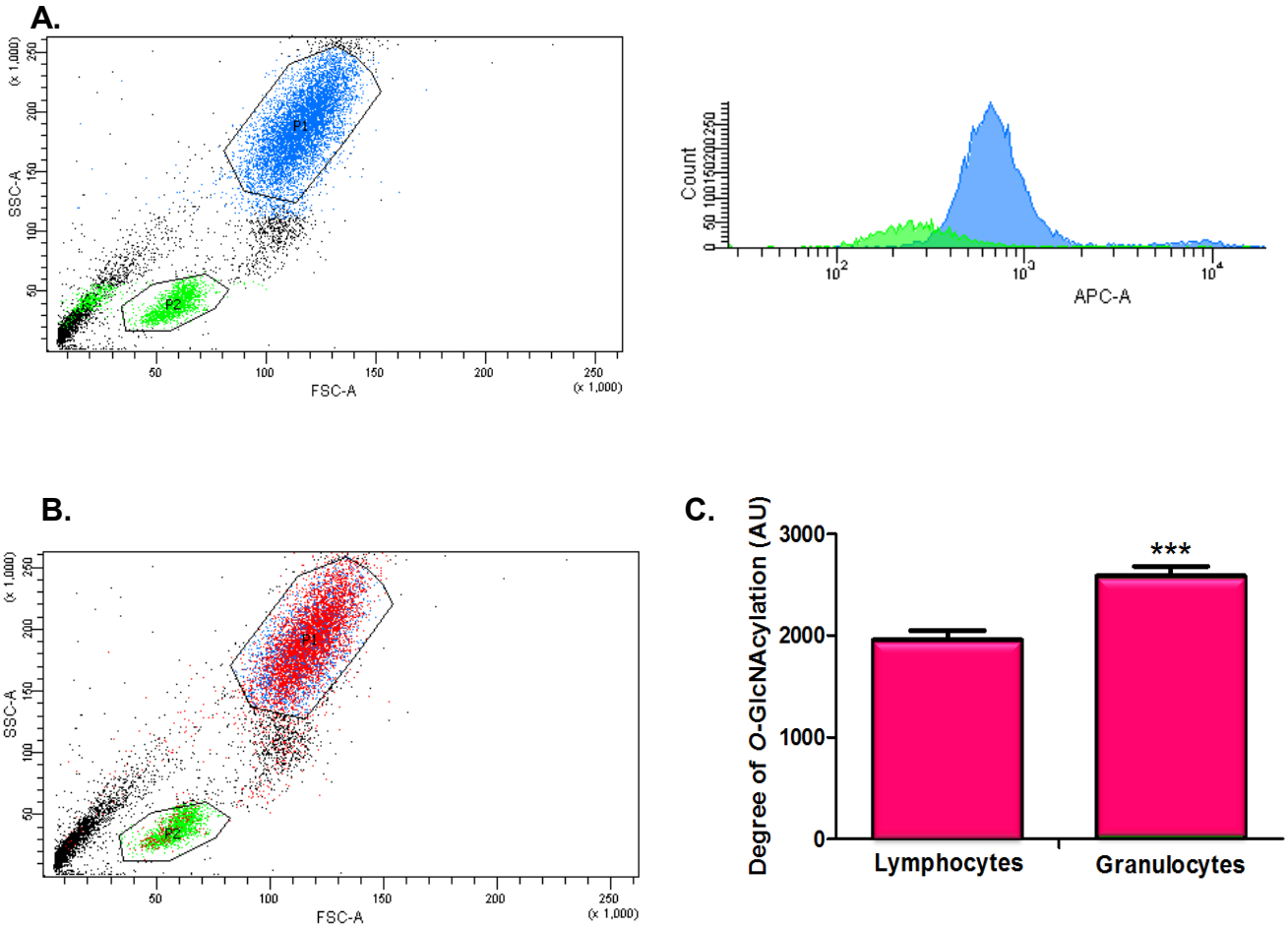


Figure 3.1 Diverse physical properties and differential CD45 fluorescence allow for the identification of leukocyte sub-populations (flow cytometry).
A. A representative histogram illustrating a total leukocyte population separated into two gated leukocyte sub-types based on forward light scatter (FSC) and linear 90° light scatter (SSC) properties (lymphocytes: green, granulocytes: blue). **B.** The same cell population (refer A above) plotted as side scatter (SSC) versus CD45 intensity. Variable CD45 fluorescence highlights the two gated leukocyte populations. **C.** The forward scatter (FSC) and side scatter (SSC) of the sample reflecting differences in the mean intensity of CD45 (red).

3.2 Differential *O*-GlcNAc modification between different leukocyte populations

The accurate distinction between leukocyte sub-populations allowed us to investigate the relative degree of *O*-GlcNAcylation in lymphocytes compared to granulocytes. Here we found a greater *O*-GlcNAc fluorescence intensity in the granulocytes (blue peak shifted far to the right) compared to the lymphocyte population (green peak) (Figure 3.2A). The SSC/FSC scatter plot confirmed this and

demonstrated increased *O*-GlcNAc signal (red) in granulocytes (blue) compared to lymphocytes (green) (Figure 3.2B). Quantification of these data showed that *O*-GlcNAcylation was 32.0 ± 4.9% higher in the granulocytes versus the lymphocytes ($p<0.001$)(Fig. 2C). To confirm these interesting findings, we also performed immunofluorescence microscopy analysis and found increased *O*-GlcNAc fluorescence (red) in granulocytes (Fig. 2D) versus lymphocytes (Fig. 2E).



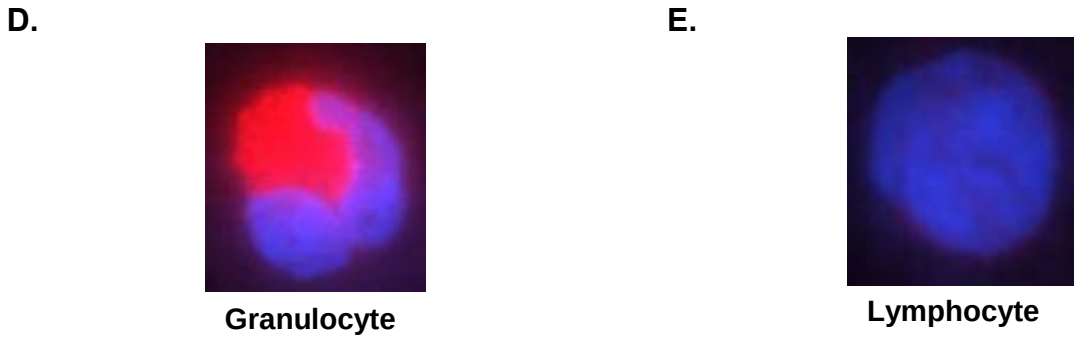


Figure 3.2 Differential display of *O*-GlcNAc signal between different leukocyte populations (flow cytometry and immunofluorescence microscopy).
A:Leukocyte histograms and corresponding scatter plot in granulocytes (blue peak) compared to lymphocytes (green peak). **B.** The forward scatter (FSC) and side scatter (SSC) reflecting *O*-GlcNAc fluorescence (red) in granulocytes (blue) compared to lymphocytes (green). **C.** Bar graph representing mean intensity of *O*-GlcNAc staining in granulocytes versus lymphocytes (n=74, ***p<0.001). **D.** Representative image of neutrophil granulocyte (blue) stained for *O*-GlcNAc (red). **E.** Representative image of lymphocyte (blue) stained for *O*-GlcNAc (red) (magnification: 60 x). AU: Arbitrary units.Values are displayed as mean ± SEM.

3.2 Similar OGA expression between different leukocyte populations

We also analyzed the relative OGA expression in the different leukocyte populations. Here we essentially found identical OGA fluorescence intensity in the granulocytes (blue peak) compared to the lymphocyte population (green peak) (Figure 3.2A). The SSC/FSC scatter plot confirmed this and demonstrates similar OGA signal (red) in granulocytes (blue) and lymphocytes (green) (Figure 3.2B). Quantification of these data showed that OGA differed by less than 0.5% between leukocyte sub-populations (Fig. 2C). Immunofluorescence microscopy analysis confirmed these findings as no clear differences in OGA fluorescent intensity were visible (refer Figures 3.3D and 3.3E for a representative image of OGA staining in granulocytes and lymphocytes, respectively).

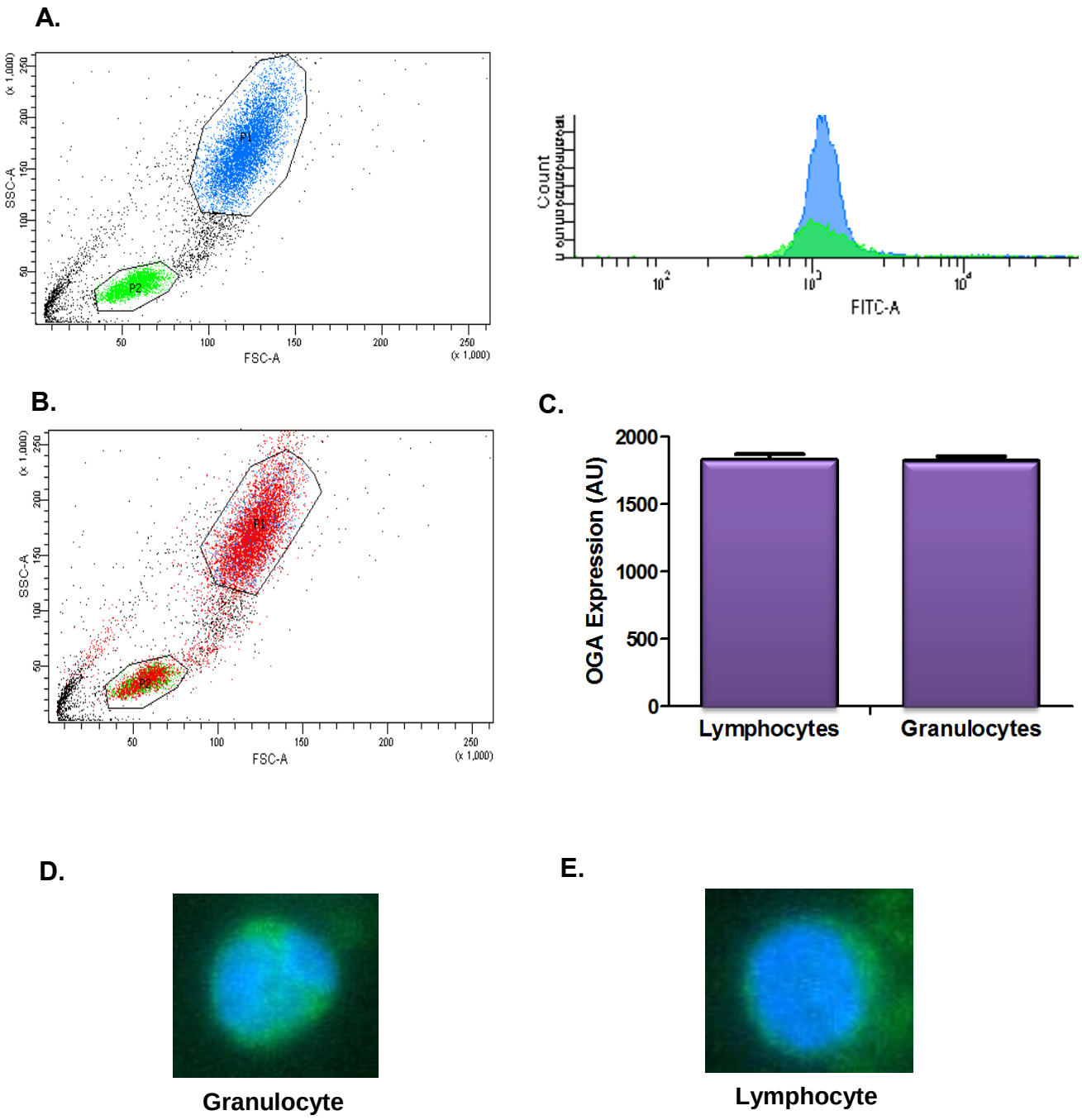


Figure 3.3 Identical display of OGA signal between different leukocyte populations (flow cytometry and immunofluorescence microscopy).
A. Leukocyte histograms and corresponding scatter plot in granulocytes (blue peak) compared to lymphocytes (green peak). **B.** The forward scatter (FSC) and side scatter (SSC) reflecting OGA fluorescence (red) in granulocytes (blue) compared to lymphocytes (green). **C.** Bar graph representing mean intensity of OGA staining in granulocytes versus lymphocytes (n=74). **D.** Representative image of neutrophil granulocyte (blue) stained for OGA (green). **E.** Representative image of lymphocyte (blue) stained for OGA (green) (magnification: 60x). AU: Arbitrary units. Values are displayed as mean ± SEM.

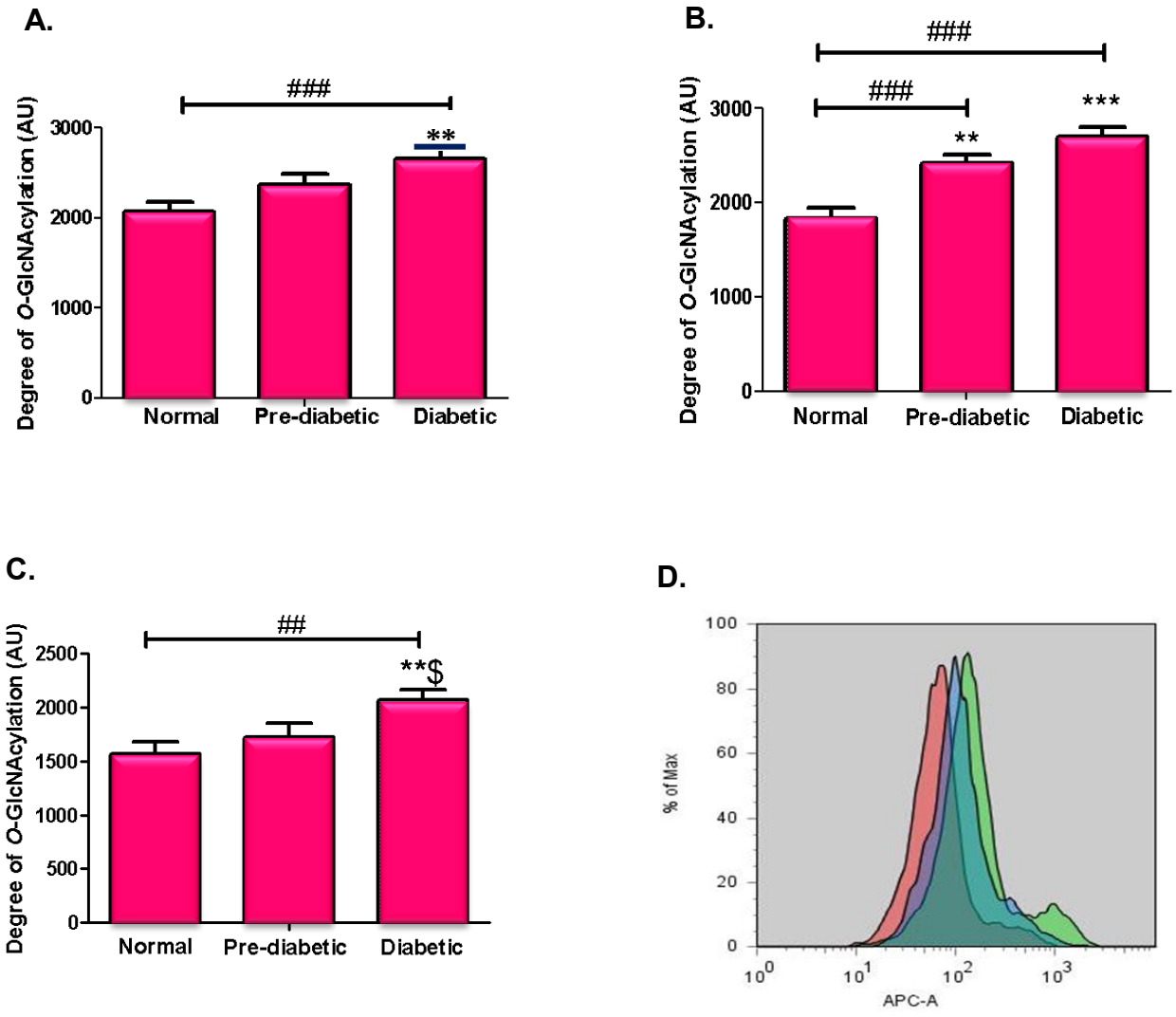
3.3 Determination of *O*-GlcNAcylation by means of flow cytometry and immunofluorescence miscroscopy

We next investigated the degree of *O*-GlcNAc modified proteins in the leukocytes of normal, pre-diabetic and diabetic individuals. Individuals were characterized as normal, pre-diabetic or diabetic according to 3.3.1) ADA FPG levels, 3.3.2) WHO FPG criteria, and 3.3.3) HbA1c levels.

3.3.1 Increases in leukocyte *O*-GlcNAcylation with increased fasting blood glucose levels (ADA)

Subjects characterized according to ADA criteria showed increased *O*-GlcNAcylation in their total leukocyte sample. Diabetic individuals exhibited an enhanced *O*-GlcNAcylation of 28.9 ± 6.2% versus normal subjects ($p<0.01$)(Figure 3.4A). However, we found no significant changes between pre-diabetic and normal subjects (Figure 3.4A). When independently examining granulocytes, the differences in *O*-GlcNAcylation were more prominent, and significant increases of 32.4 ± 4.4% and 47.6 ± 5.8% were found in individuals with pre-diabetes and diabetes, respectively (Figure 3.4B). Lymphocytes similarly showed a robust increase in *O*-GlcNAc intensity for diabetic individuals (32.4 ± 5.8%)($p<0.01$), while there was a significant difference between pre-diabetic and diabetic lymphocytes (20.6 ± 5.3%) (Figure 3.4C). These findings were confirmed with our population fluorescence data, where a greater mean *O*-GlcNAc fluorescence intensity was observed in the pre-diabetic group (blue peak shifted to the right), indicating augmented *O*-GlcNAcylation. Moreover, we also observed greater *O*-GlcNAcylation between pre-diabetic and diabetic individuals (green peak shifted to the right)

(Figure 3.34E). Our flow cytometric data were confirmed with immunofluorescence microscopy where the fluorescent signal was quantified in the total leukocyte population (Figure 3.4E). Representative images illustrate greater *O*-GlcNAc signal (red) in the leukocytes (blue) of diabetic subjects compared to healthy individuals (Figure 3.4F).



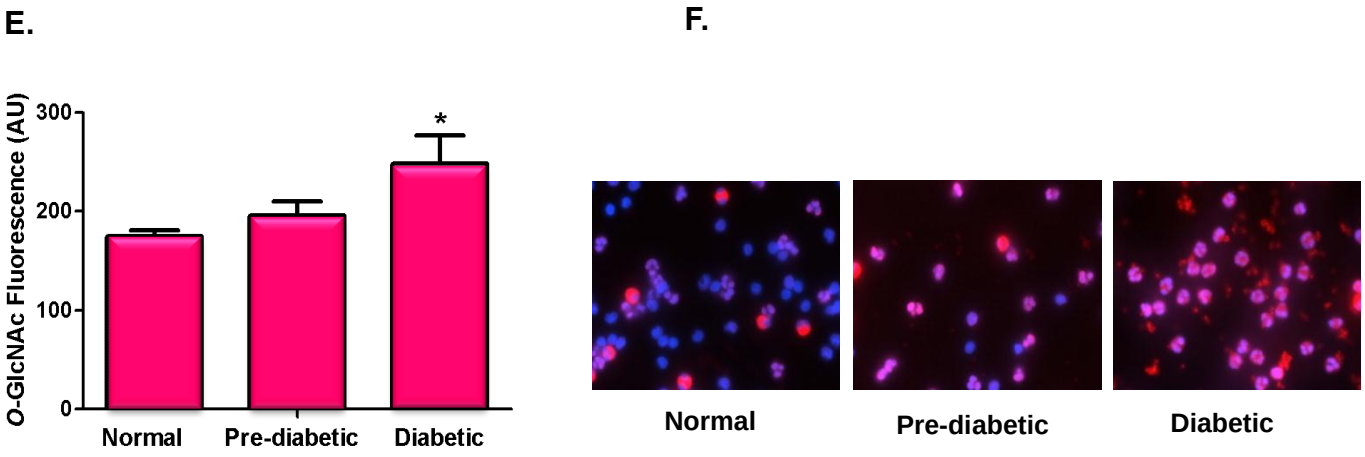


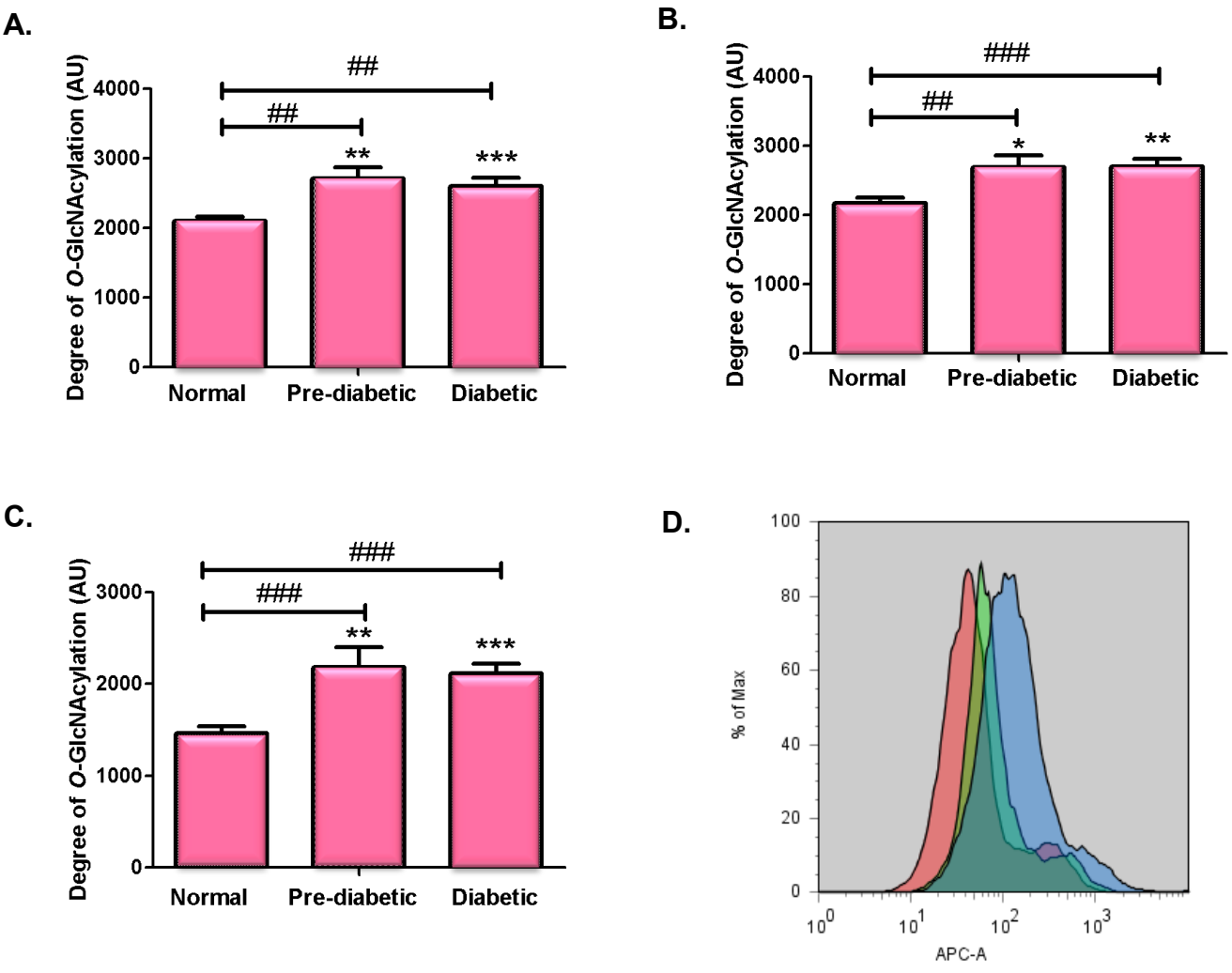
Figure 3.4 Increased *O*-GlcNAcylation of leukocyte proteins in ADA characterized pre-diabetic and diabetic individuals (flow cytometry and immunofluorescence microscopy).

A: *O*-GlcNAcylation in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=62, ###p<0.001, **p<0.01 vs. normal group). **B:** Granulocyte *O*-GlcNAcylation levels (n=51, ##p<0.01, ###p<0.001, **p<0.01, ***p<0.001 vs. normal group). **C:** Lymphocyte *O*-GlcNAcylation levels (n=54, ##p<0.01, $p<0.05 vs. pre-diabetic group, **p<0.01 vs. normal group). **D:** Peak shifts of total leukocyte population fluorescence data show *O*-GlcNAcylation for normal (red), pre-diabetic (blue) and diabetic individuals (green) **E:** Quantification of *O*-GlcNAc signal intensity with immunofluorescence microscopy in the total leukocyte population (n=25, *p<0.05 vs. normal group). **F:** Representative images from immunofluorescence microscopy performed for pre-diabetic, diabetic and normal individuals. Leukocytes were stained for *O*-GlcNAc (red) and Hoechst dye (blue) (magnification: 60x). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.3.2 Increased *O*-GlcNAc modification with elevated fasting blood glucose concentrations (WHO)

O-GlcNAc modified protein levels were next investigated in individuals characterized by WHO guidelines. Here, significant increases in *O*-GlcNAcylation were found in individuals with both pre-diabetes and diabetes, respectively (Figure 3.5A). However, we found no significant changes between pre-diabetic and diabetic subjects (Figure 3.5A). For granulocytes, we found that *O*-GlcNAc modified protein levels were higher in pre-diabetic (24.4 ± 7.8%)($p<0.05$), as well as in diabetic leukocytes (24.7 ± 4.9%)($p<0.01$), versus the normal group (Figure 3.5B). This result was intensified in the lymphocytes as increases in *O*-GlcNAc modification of

49.1.4 ± 15.1% ($p<0.01$) and 44.9 ± 6.9% ($p<0.001$) were found in pre-diabetic and diabetic individuals, respectively (Figure 3.5C). These findings were confirmed with our population fluorescence data, where a greater mean *O*-GlcNAc fluorescence intensity was observed in the pre-diabetic group (blue peak shifted to the right), compared to the normal group (red peak) (Figure 3.5D). Our flow cytometric data were supported by immunofluorescence microscopy where *O*-GlcNAc fluorescent intensity was quantified in the total leukocyte population. However, although changes were observed, it did not reach statistical significance (Figure 3.5E). Representative images display a greater *O*-GlcNAc signal (red) in the leukocytes (blue) of diabetic subjects compared to healthy individuals (Figure 3.5F).



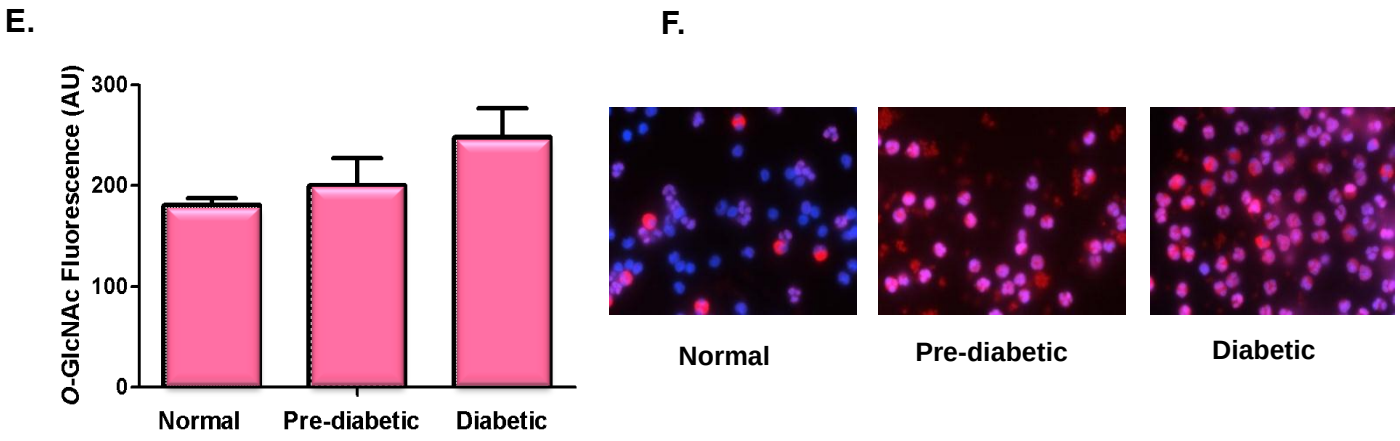


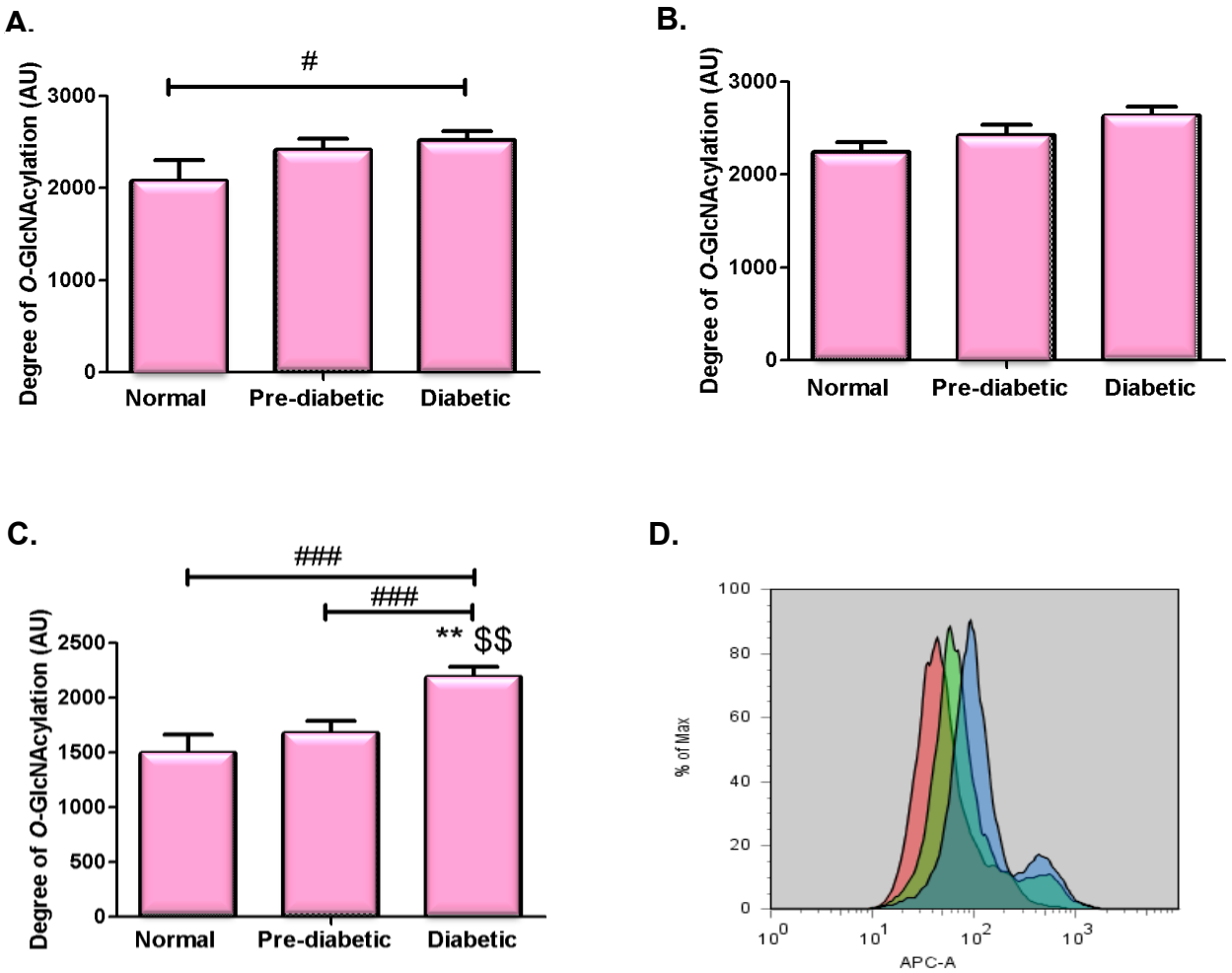
Figure 3.5 Increased *O*-GlcNAcylation of leukocyte proteins in WHO characterized pre-diabetic and diabetic individuals (flow cytometry and immunofluorescence microscopy).

A: *O*-GlcNAcylation in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=62, ##P<0.01, ***p<0.001, **p<0.01 vs. normal group). **B:** Granulocyte *O*-GlcNAcylation levels (n=48, ##p<0.01, ###p<0.001, **p<0.01, *p<0.05 vs. normal group). **C:** Lymphocyte *O*-GlcNAcylation levels (n=53, ###P<0.001, ***p<0.001, **p<0.01 vs. normal group). **D:** Peak shifts of total leukocyte population fluorescence data show *O*-GlcNAcylation for normal (red), pre-diabetic (green) and diabetic individuals (blue) **E:** Quantification of *O*-GlcNAc signal intensity with immunofluorescence microscopy in the total leukocyte population (n=25, no significant differences). **F:** Representative images from immunofluorescence microscopy performed for pre-diabetic, diabetic and normal individuals. Leukocytes were stained for *O*-GlcNAc (red) and Hoechst dye (blue) (magnification: 60x) AU: Arbitrary units. Values are expressed as mean ± SEM.

3.3.3 Leukocyte *O*-GlcNAcylation increases with rising HbA1c levels

When next characterized our study subjects according to HbA1c levels and therefore determined their respective *O*-GlcNAcylation levels. For the total leukocyte sample we recorded an elevation of 21.3 ± 4.7% in the mean intensity of *O*-GlcNAc between normal and diabetic samples ($p<0.05$)(Figure 3.6A). Although slight increases were observed, granulocyte *O*-GlcNAcylation yielded no significant differences (Figure 3.6B). However, lymphocytes displayed robust increases in *O*-GlcNAc intensity for diabetic individuals (46.3 ± 6.2%)($p<0.001$). Additionally, a significant difference in lymphocyte *O*-GlcNAc levels were observed between pre-diabetic and diabetic lymphocytes (30.6 ± 5.5%) (Figure 3.6C). These findings were

confirmed by our population fluorescence data, where a greater mean *O*-GlcNAc fluorescence intensity was observed in the pre-diabetic group (blue peak shifted to the right), compared to the normal group (red peak) (Figure 3.6D). Immunofluorescence microscopy data showed a slight increase between pre-diabetic and diabetic individuals (Figure 3.6E), although this was not statistically significant. Representative images display a greater *O*-GlcNAc signal (red) in the leukocytes (blue) of diabetic subjects compared to pre-diabetic individuals (Figure 3.6F).



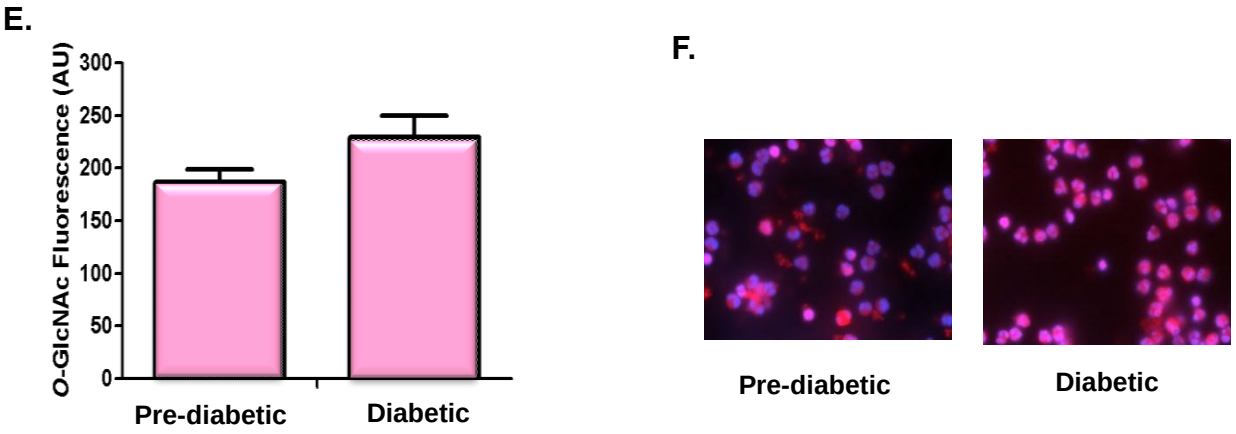


Figure 3.6 Increased *O*-GlcNAc modifications in pre-diabetic and diabetic leukocytes when characterized according to HbA1c levels (flow cytometry and immunofluorescence microscopy).

A: *O*-GlcNAcylation in total WBC population-prediabetic and diabetic subjects versus healthy individuals (n=57, $^{\#}p<0.05$).**B:** Granulocyte *O*-GlcNAcylation levels (n=51, no significant differences observed). **C:** Lymphocyte *O*-GlcNAcylation levels (n=54, $^{\#\#\#}p<0.001$, $^{\$\$}p<0.01$ vs. pre-diabetic group, $^{**}p<0.01$ vs. normal group). **D:** Peak shifts of total leukocyte population fluorescence data show *O*-GlcNAcylation for normal (red), pre-diabetic (green) and diabetic individuals (blue) **E:** Quantification of *O*-GlcNAc signal intensity (immunofluorescence microscopy) in the total leukocyte population between pre-diabetic and diabetic individuals (n=24, no significant differences). **F:** Representative images from immunofluorescence analysis performed for pre-diabetic and diabetic individuals. Leukocytes were stained for *O*-GlcNAc (red) and Hoechst dye (blue) (magnification: 60 x). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.4 Determination of *O*-GlcNAcylation by Western blotting

To support our flow cytometric and immunofluorescence data, we additionally analyzed global *O*-GlcNAcylation levels by Western blotting (Figure 3.7A). Here total leukocyte *O*-GlcNAc protein levels were increased in the diabetic samples compared to the normal groups (Figure 3.7B). Although marked increases were observed between normal and pre-diabetic and pre-diabetic and diabetic leukocytes, these differences were not statistically significant (Figure 3.7B).

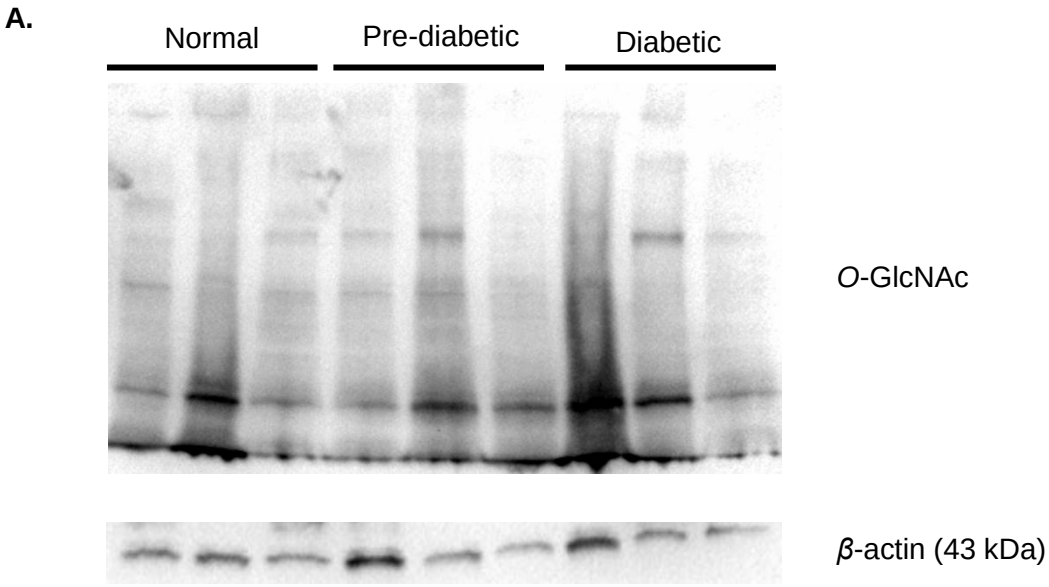


Figure 3.7 Western blot analysis of *O*-GlcNAcylation in normal, pre-diabetic and diabetic individuals.

A: Representative *O*-GlcNAc blot **B:** Bar graphs show volumetric quantification of *O*-GlcNAc levels in pre-diabetic and diabetic groups versus the normal group (normalized to β-actin)(n= normal [9], pre-diabetic [15], diabetic [12]; #p<0.05). AU: Arbitrary units. Values are expressed as mean ± SEM.

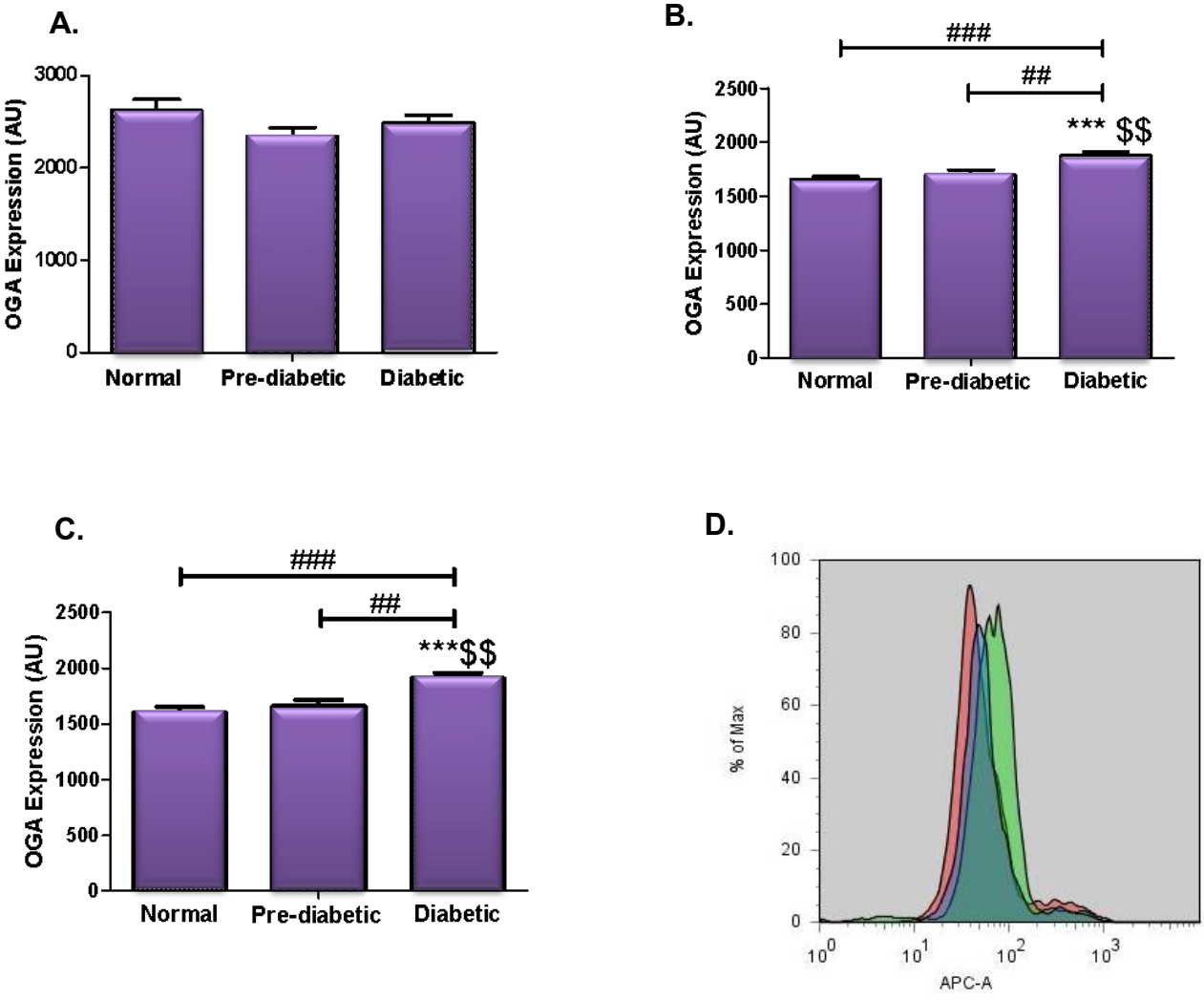
3.5 Investigation of OGA expression by flow cytometry and immunofluorescence miscroscopy

Here we investigated OGA protein levels in the leukocytes of normal, pre-diabetic and diabetic individuals. Individuals were once again characterized as normal, pre-diabetic or diabetic according to 3.5.1) ADA FPG levels, 3.5.2) WHO FPG criteria, and 3.5.3) HbA1c levels.

3.5.1 Differential expression of *O*-GlcNAcase (OGA) between healthy, pre-diabetic and diabetic individuals (ADA characterized)

These OGA expression data exhibits a moderate decrease (not statistically significant) in the total leukocyte population of pre-diabetic individuals (Figure 3.8A). Moreover, OGA protein levels were modestly increased in diabetic individuals (not statistically significant) versus the pre-diabetic group (Figure 3.8A). Granulocytes displayed a 13.3 ± 2.4% ($p<0.001$) higher OGA expression in diabetic subjects and could significantly distinguish between pre-diabetic and diabetic subjects (10.9 ± 2.3%) (Figure 3.8B). An identical pattern was observed in the lymphocytes where even greater differences were found compared to granulocytes. Here lymphocyte OGA expression increased by 19.3 ± 3.1% ($p<0.001$) in diabetic subjects, and was significantly different between pre-diabetic and diabetic subjects (14.9 ± 3%) (Figure 3.8C). The mean OGA fluorescence intensities demonstrated increased OGA expression in the diabetic leukocytes (green peak) compared to the normal group (red peak) (Figure 3.8D). OGA protein levels were also determined by fluorescence microscopy where a modest increase was observed in diabetic individuals. However, this was not statistically significant (Figure 3.8E).

Representative images display a markedly increased green fluorescent signal (OGA expression) in the leukocytes of diabetic subjects (Figure 3.8F).



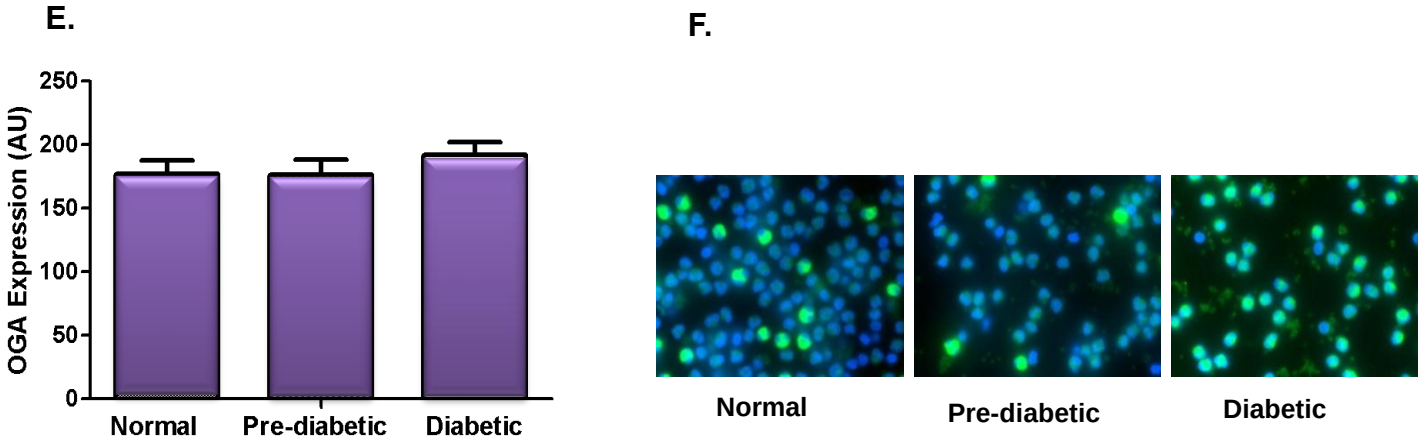


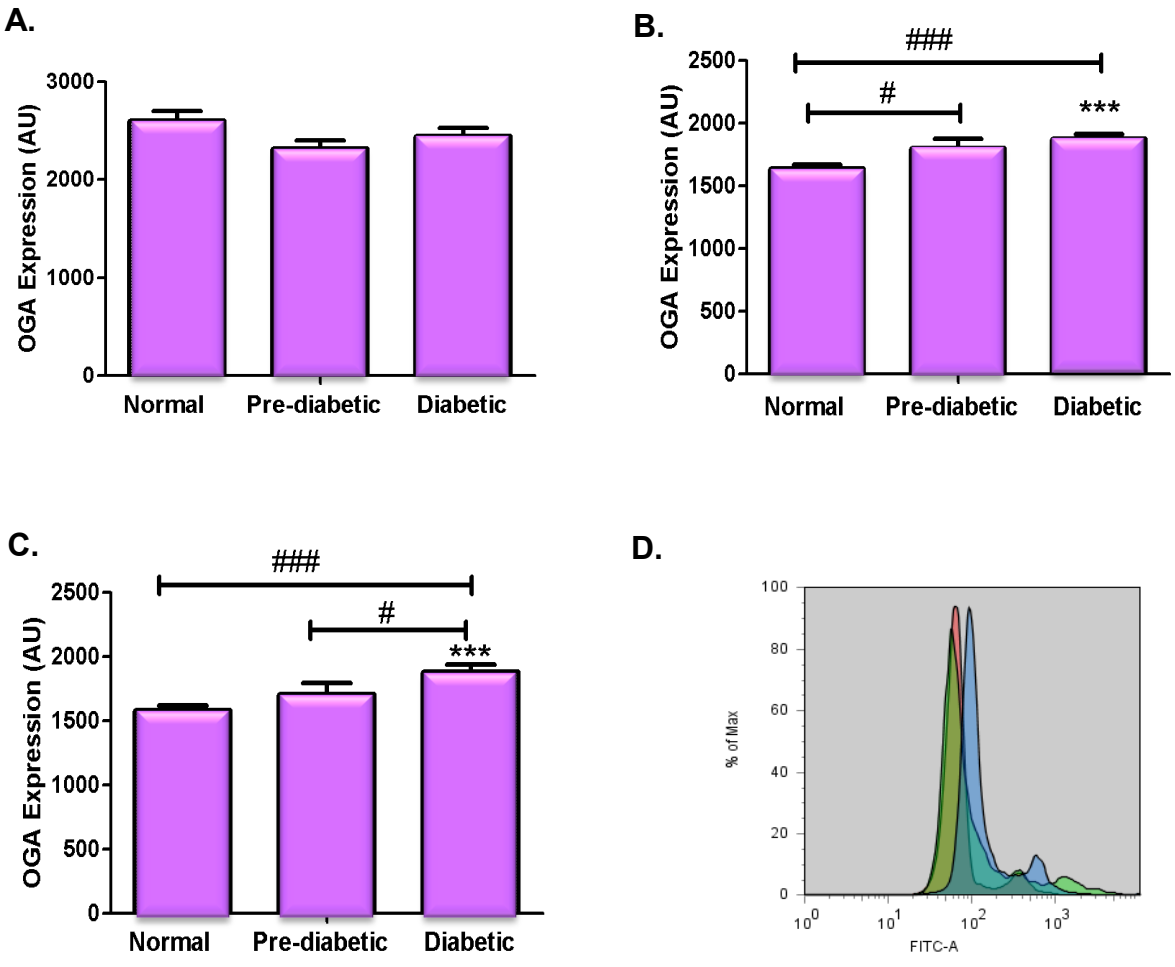
Figure 3.8 Differential leukocyte OGA protein expression in ADA-defined pre-diabetic and diabetic individuals (flow cytometry and immunofluorescence microscopy).

A: OGA expression in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=62). **B:** OGA protein levels in granulocytes (n=56, $^{\#\#}$P<0.01, $^{\#\#\#}$p<0.001, $^{\$\$}$p<0.01 compared to pre-diabetic group, ***p<0.001 vs. normal group). **C:** OGA expression in lymphocytes (n=49, $^{\#\#}$P<0.01, $^{\#\#\#}$p<0.001, $^{\$\$}$p<0.01 compared to pre-diabetic group, ***p<0.001 vs. normal group). **D:** Peak shifts of total leukocyte population for healthy (red peak), pre-diabetic (blue peak) and diabetic subjects (green peak).**E:** Quantification of OGA fluorescent intensity in the total leukocyte population between normal, pre-diabetic and diabetic individuals (n=25, no significant differences). **F:** Representative images from immunofluorescence microscopy. Leukocytes were stained for OGA (green) and Hoechst dye (blue) (magnification: 60 x). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.5.2 Differential OGA expression between normal, pre-diabetic and diabetic subjects (WHO characterized)

We next investigated OGA expression in individuals characterized by the diagnostic criteria of the WHO. We found no significant differences in OGA protein levels for total leukocyte sample (Figure 3.9A). OGA expression was, however, increased in the granulocyte population, i.e. increases of 10 ± 4.5% (p<0.05) and 14.3 ± 4.5% (p<0.001) was observed between the normal and pre-diabetic group, and between normal and diabetic individuals, respectively. There was no statistical significance between the pre-diabetic and diabetic groups (Figure 3.9B). Elevated OGA expression was also observed in the lymphocytes, with an increase of 18.8 ± 3.4%

($p<0.001$) found in diabetic samples compared to normal individuals. Lymphocyte OGA data could additionally distinguish between pre-diabetes and diabetes, with an increase of 10.2 ± 3.2% ($p<0.05$) observed in the diabetic group versus pre-diabetic individuals (Figure 3.9C). The mean OGA fluorescence intensities demonstrated increased OGA expression in the diabetic leukocytes (green peak) compared to the healthy leukocytes (red peak) (Figure 3.9D). The fluorescent intensity of OGA was determined and quantified using immunofluorescence microscopy (total leukocyte sample) and a slight increase (not statistically significant) was observed in the diabetic group (Figure 3.9E). Representative images display elevated OGA fluorescent signal (green) in the diabetic leukocytes (Figure 3.9F).



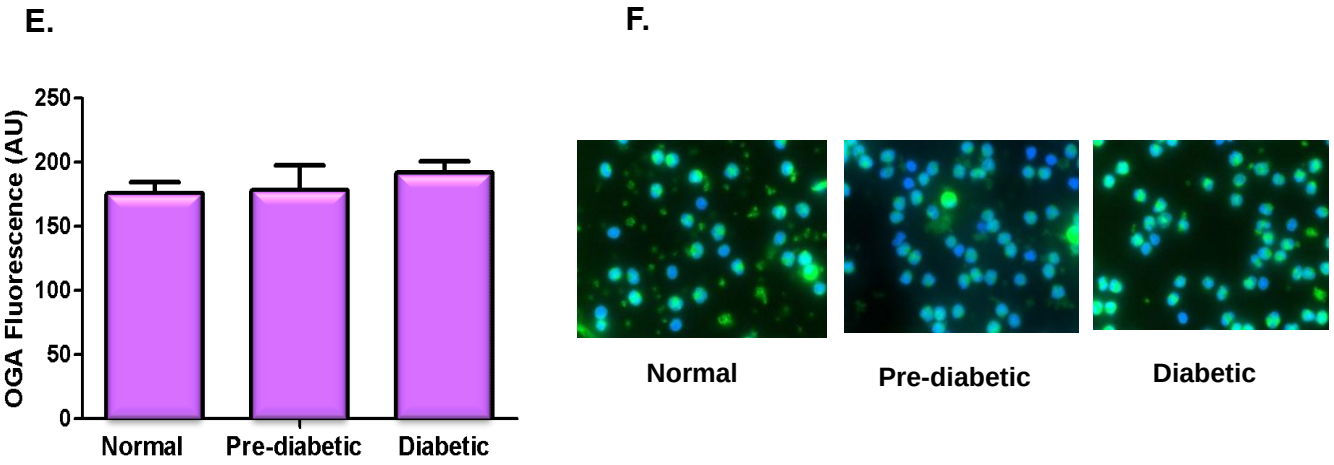


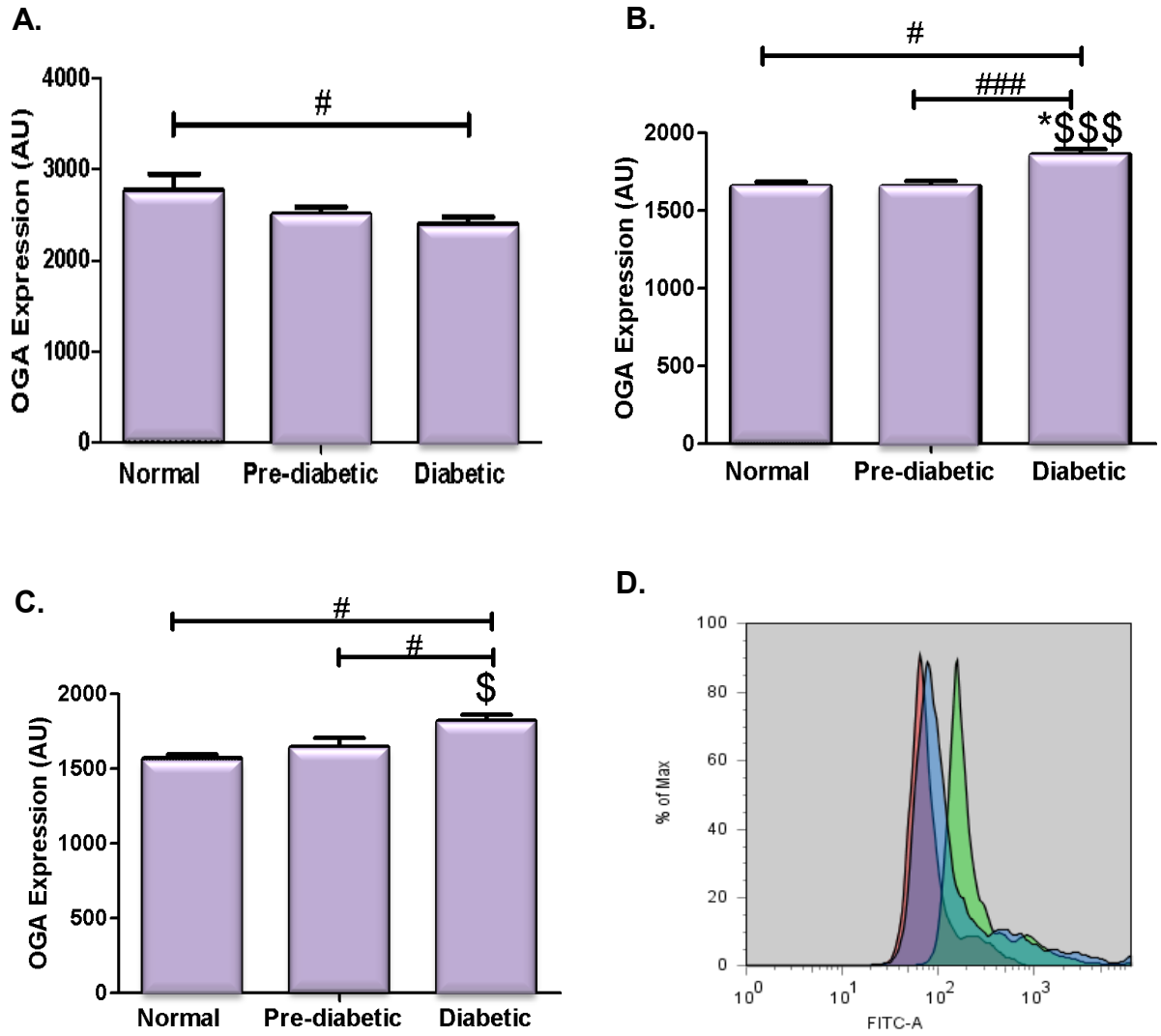
Figure 3.9 Differential leukocyte OGA protein expression in WHO defined pre-diabetic and diabetic individuals (flow cytometry and immunofluorescence microscopy).

A: OGA expression in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=61). **B:** OGA expression in granulocytes (n=57, ###p<0.001, #p<0.05, ***p<0.001 vs. normal group). **C:** OGA protein levels in lymphocytes (n=50, ###p<0.001, #p<0.05, ***p<0.001 vs. normal group). **D:** Peak shifts of total leukocyte population for healthy (red peak), pre-diabetic (green peak) and diabetic subjects (blue peak).**E:** Quantification of OGA fluorescent intensity in the total leukocyte population between normal, pre-diabetic and diabetic individuals (n=26, no significant differences). **F:** Representative images from immunofluorescence microscopy. Leukocytes were stained for OGA (green) and Hoechst dye (blue) (magnification: 60 x). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.5.3 OGA is differentially expressed between normal, pre-diabetic and diabetic subjects when characterized by HbA1c levels

When participants were categorized according to HbA1c levels, we found that diabetic individuals presented with a reduction in leukocyte OGA expression (13.4 ± 2.8%) ($p<0.05$) (Figure 3.10A). Granulocytes displayed higher OGA expression in diabetic subjects (12.6 ± 2.1%) ($p<0.05$) and distinguished between pre-diabetic and diabetic subjects (12.5 ± 2.1%) ($p<0.001$) (Figure 3.10B). Likewise, OGA expression in lymphocytes of diabetic subjects was significantly elevated (16 ± 2.5%) and slightly more sensitive than for granulocytes. For the latter we also found a significant difference between pre-diabetic and diabetic subjects (Figure 3.10C). Population fluorescence data show increased OGA fluorescence intensities

in the diabetic leukocytes (green peak) (Figure 3.10D). OGA protein levels were also determined using fluorescence microscopy and OGA signal was quantified in pre-diabetic and diabetic blood samples only. A modest increase was observed in the diabetic individuals (not significant) (Figure 3.10E). Representative images support flow cytometry results, i.e. green fluorescent signal (OGA expression) augmented in the leukocytes of diabetic subjects compared to pre-diabetic individuals (Figure 3.10F).



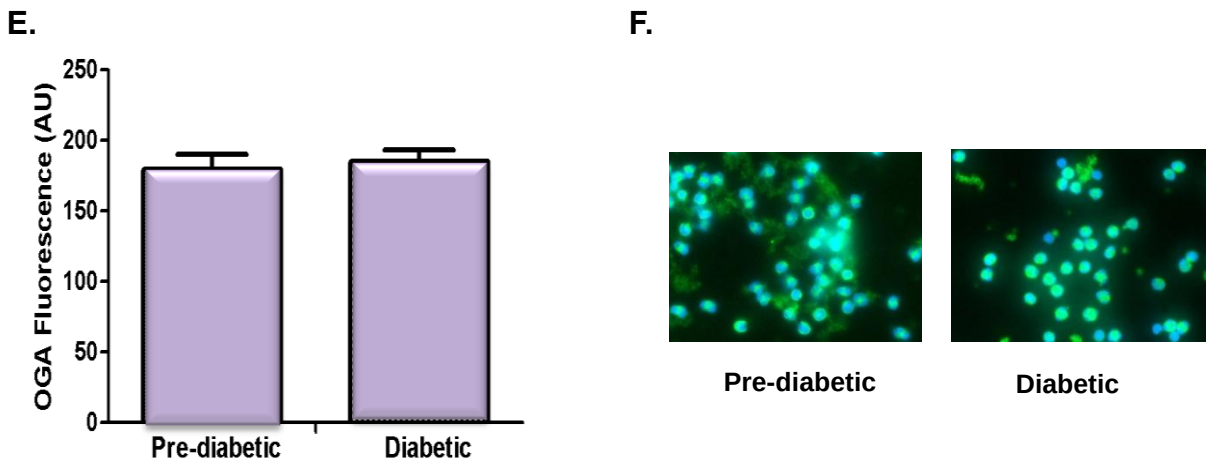


Figure 3.10 Differential leukocyte OGA protein expression in pre-diabetic and diabetic individuals characterized by HbA1c levels (flow cytometry and immunofluorescence microscopy).

A: OGA expression in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=59, $^{\#}p<0.05$)). **B:** OGA protein levels in granulocytes (n=50, $^{\#\#\#}p<0.001$, $^{\#}p<0.05$, $^{\$\$\$}p<0.001$ compared to pre-diabetic group, *p<0.05 vs. normal group). **C:** OGA expression in lymphocytes (n=44, $^{\#}p<0.05$, $^{\$}p<0.05$ compared to pre-diabetic group). **D:** Peak shifts of total leukocyte population for healthy (red peak), pre-diabetic (blue peak) and diabetic subjects (green peak).**E:** Quantification of OGA signal intensity in the total leukocyte population between pre-diabetic and diabetic individuals (n=25, no significant differences). **F:** Representative images from immunofluorescence microscopy. Leukocytes were stained for OGA (green) and Hoechst dye (blue) (magnification: 60 x). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.6 Evaluation of OGA expression by Western blotting

Here we aimed to support our findings by determining the OGA protein levels in normal, pre-diabetic and diabetic individuals by Western blotting analysis (Figure 3.11A). Volumetric quantification detected a highly significant increase in OGA expression in the pre-diabetic individuals versus normal individuals. Additionally, a significant increase was observed in the diabetic participants versus the normal group. However, an unexpected decrease in OGA protein levels was observed in the diabetic group versus the pre-diabetic group (Figure 3.11B).

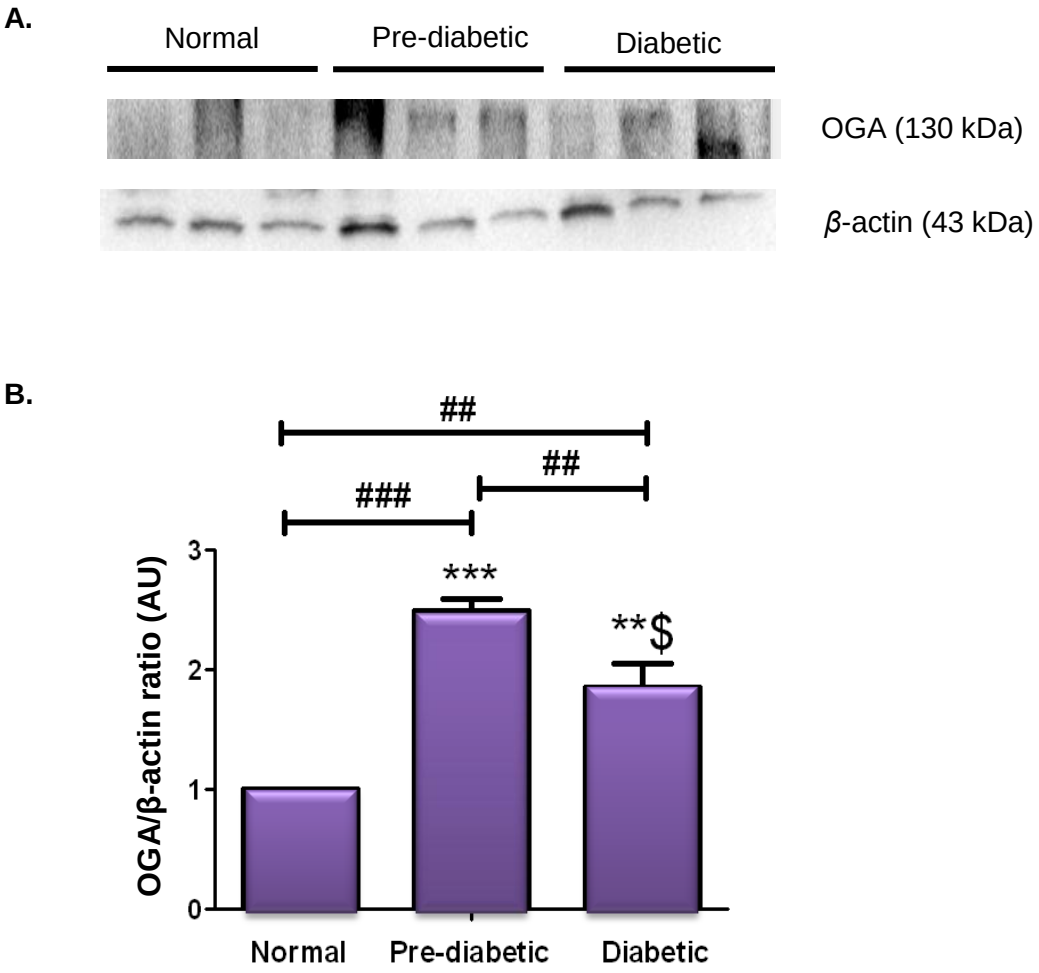


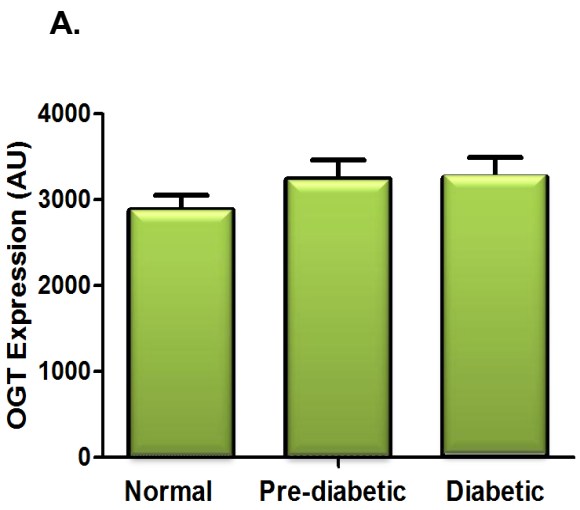
Figure 3.11 OGA western blot analysis in normal, pre-diabetic and diabetic individuals.

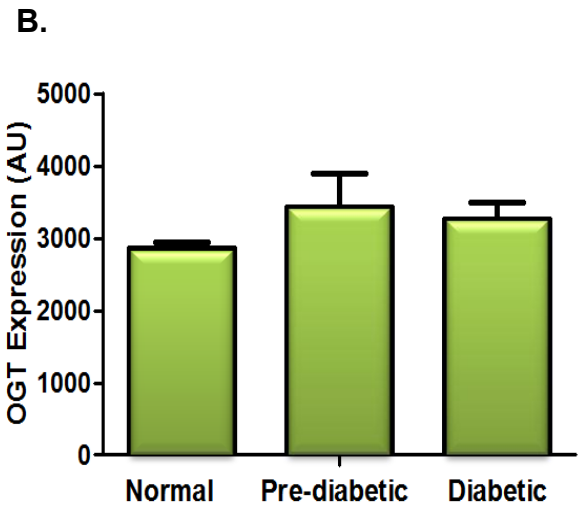
A: Representative OGA blot **B:** Bar graphs show volumetric quantification of OGA protein levels in pre-diabetic and diabetic groups versus the normal group (normalized to β-actin)(n= normal [4], pre-diabetic [5], diabetic [5]; $^{###}p<0.001$, $^{##}p<0.01$, $^{\$}p<0.05$ compared to pre-diabetic group, $^{***}p<0.001$, $^{**}p<0.01$ compared to normal group). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.7 Investigation of OGT expression by flow cytometry and immunofluorescence microscopy

We additionally measured OGT protein levels using flow cytometry and immunofluorescence microscopy. However, unlike *O*-GlcNAc and OGA determinations, we experienced great technical difficulties to consistently quantify OGT levels. As a result of the poor reproducibility, the sample numbers available for analysis were very small. Due to the variability and unreliable nature of the OGT antibody, we did not quantify OGT expression in leukocyte sub-populations, nor did we have a sample size big enough to quantify OGT protein levels with immunofluorescence microscopy.

We did, however, investigate the expression of OGT in the total WBC population. No significant differences in OGT expression was observed between normal, pre-diabetic or diabetic individuals when characterized by ADA guidelines (Figure 3.12A), WHO recommendations (Figure 3.12B) or HbA1c levels (Figure 3.12B). Our flow cytometric data were qualitatively confirmed by immunofluorescence microscopy, i.e. no visible differences were detected between images (Figure 3.12D).





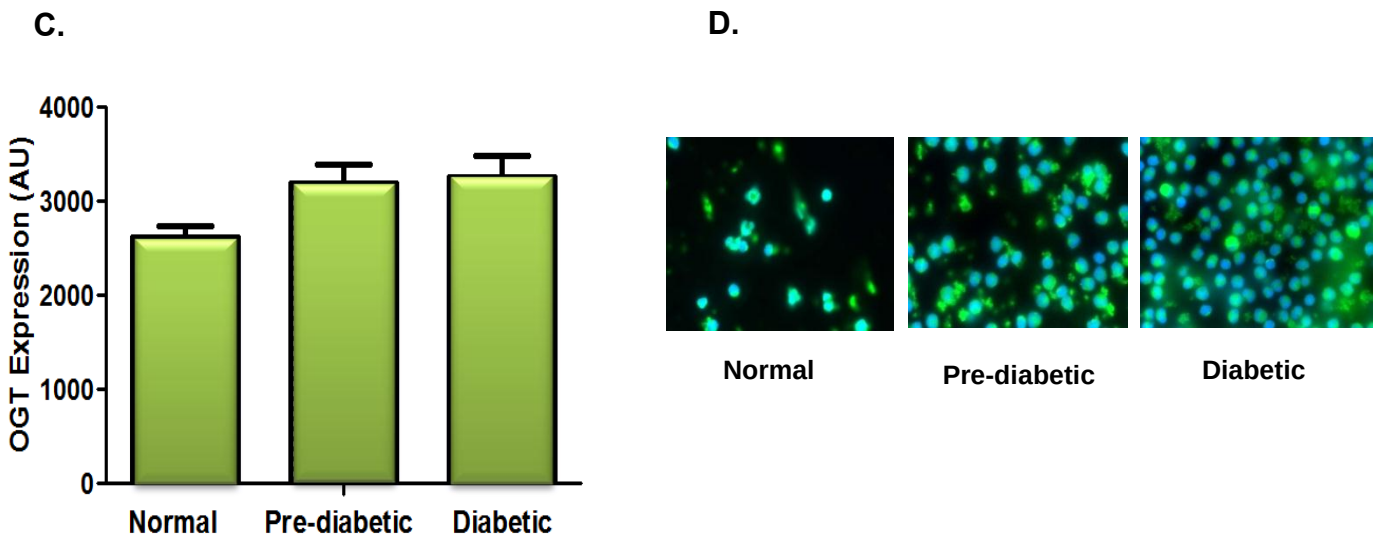
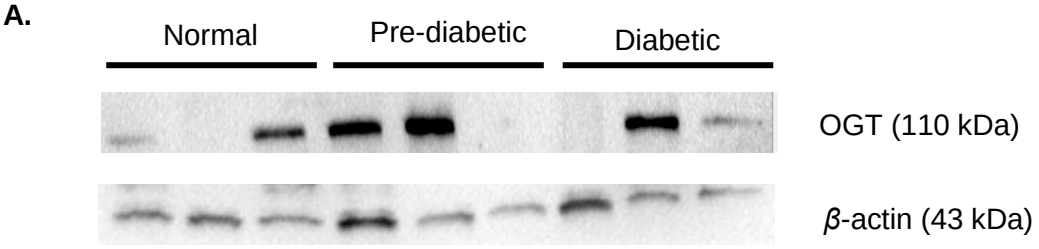


Figure 3.12 OGT expression in the total leukocyte population of normal, pre-diabetic and diabetic participants.

A: OGT protein levels in subjects characterized according to ADA criteria (n=24). **B:** The expression of OGT in participants defined as normal, pre-diabetic and diabetic according to the diagnostic guidelines of WHO (n=21). **C:** OGT expression in individuals characterized by HbA1c levels (n=27) **D:** Representative images from immunohistochemistry. Leukocytes were stained for OGT (green) and Hoechst dye (blue). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.8 Examination of OGT expression by means of Western blotting

After obtaining an alternative OGT antibody (AL 28) (Gerald Hart Laboratory, Johns Hopkins), we also investigated leukocyte OGT expression by Western blotting (Figure 3.13A). Although great variability still occurred, a significant increase in OGT protein levels were observed in the pre-diabetic individuals compared to the normal group (Figure 3.13B).



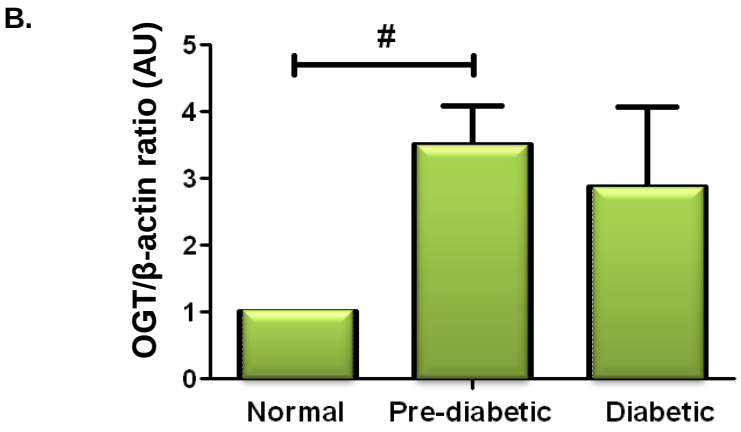


Figure 3.13 Western blot analysis of OGT protein levels in normal, pre-diabetic and diabetic individuals.

A: Representative OGT blot **B:** Bar graphs show volumetric quantification of OGT protein levels in pre-diabetic and diabetic groups versus the normal group (normalized to β-actin)(n= normal [7], pre-diabetic [8], diabetic [8]; $^{\#}p<0.05$). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.9 Determination of *O*-GlcNAc/OGA ratios

Due to the differential *O*-GlcNAcylation and OGA expression observed between normal, pre-diabetic and diabetic subjects, we decided to perform an analysis of the *O*-GlcNAc/OGA ratios for flow cytometric data. We therefore explored whether any changes in this ratio would occur between subjects when characterized by ADA criteria, WHO recommendations, and HbA1c levels.

3.9.1 Elevated *O*-GlcNAc/OGA ratios in pre-diabetic and diabetic individuals (ADA criteria)

The investigation of the *O*-GlcNAc/OGA ratio in the total leukocyte population yielded a significant increase of 32.9 ± 6.5% in the diabetic individuals versus the normal group (Figure 3.14A). The differences in *O*-GlcNAc/OGA ratio between

normal and diabetic participants were more prominent in the granulocyte population, with an increase of 31.8 ± 3.5% ($p<0.01$). Moreover, granulocytes could distinguish between normal and pre-diabetic individuals, with a difference of 28.5 ± 5.3% ($p<0.01$) detected between these two groups (Figure 3.14B). However, lymphocytes displayed decreased sensitivity with a slight but statistically insignificant increase in the diabetic group compared to the pre-diabetic and normal subjects (Figure 3.14C).

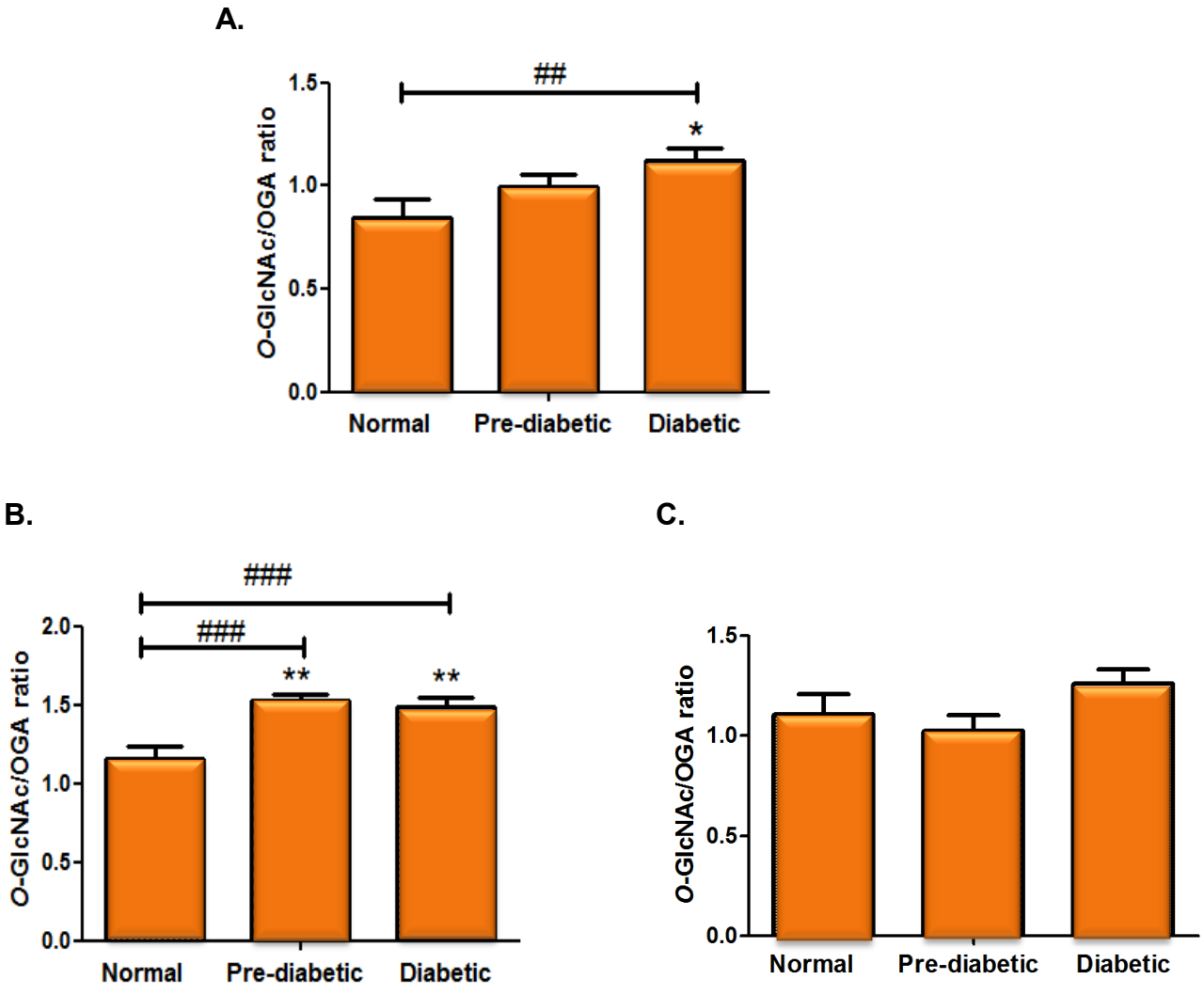


Figure 3.14 *O*-GlcNAc/OGA ratio is increased in pre-diabetic and diabetic individuals characterized by ADA diagnostic criteria (flow cytometry).

A: *O*-GlcNAc/OGA ratio in total leukocyte population of pre-diabetic and diabetic subjects versus normal individuals (n=53, ##p<0.01, *p<0.05 vs. normal group). **B:** *O*-GlcNAc/OGA ratio in granulocytes (n=50, ###p<0.001, **p<0.01 vs. normal group). **C:** Lymphocyte *O*-GlcNAc/OGA ratio (n=55). Values are expressed as mean ± SEM.

3.9.2 O-GlcNAc/OGA ratio increases in pre-diabetes and diabetes (WHO criteria)

For the total white blood cell population we found significant increases in the *O*-GlcNAc/OGA ratio of 37.1 ± 11.9% and 36.8 ± 6.6% for pre-diabetic and diabetic individuals, respectively (Figure 3.15A). No differences were observed in the granulocytes between any of the three groups (Figure 3.14B). However, in the lymphocytes, *O*-GlcNAc/OGA ratio increased in the pre-diabetic group (28.3 ± 15.5%), as well as in the diabetic group (23.9 ± 7%), compared to healthy individuals (Figure 3.15C).

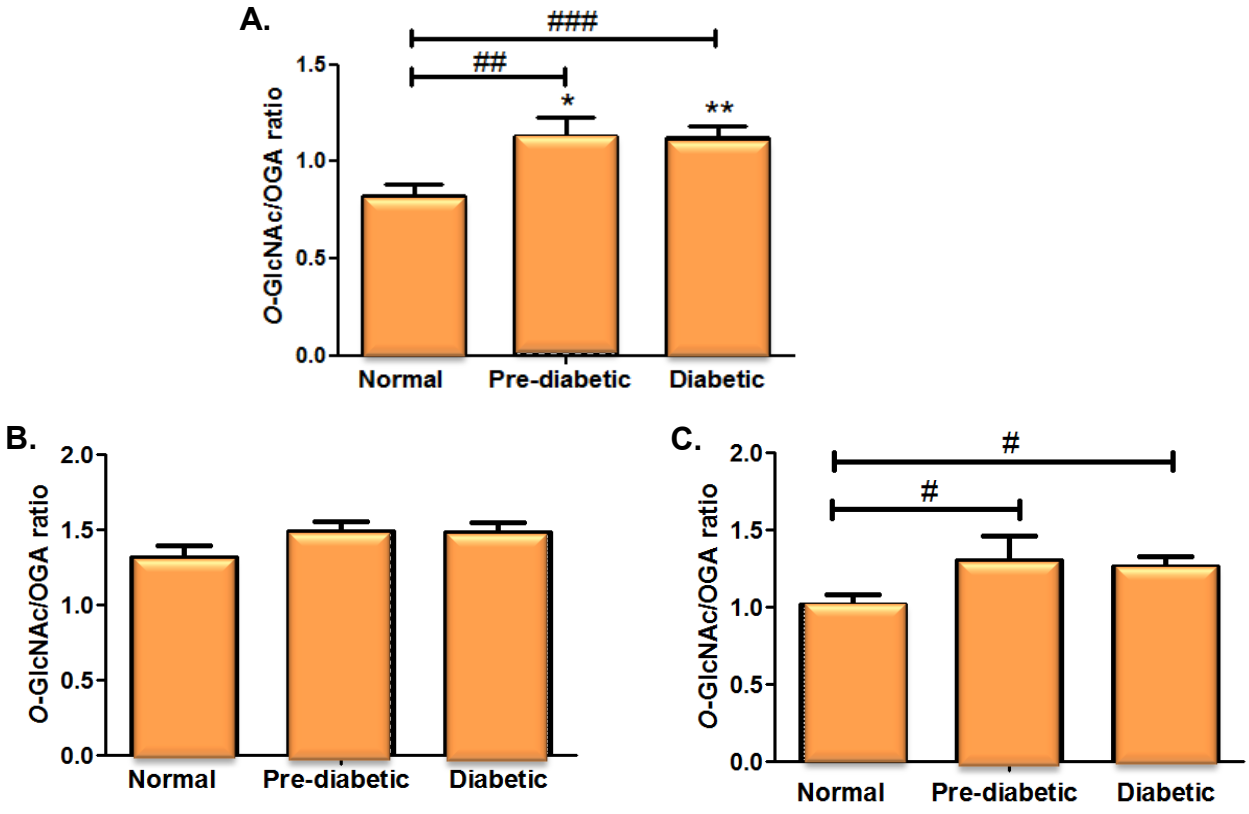


Figure 3.15 Elevated *O*-GlcNAc/OGA ratio in pre-diabetic and diabetic individuals characterized by WHO diagnostic guidelines (flow cytometry).

A: Total WBC sample's *O*-GlcNAc/OGA ratio in pre-diabetic and diabetic participants versus normal individuals (n=53, ###p<0.001, ##p<0.01, **p<0.01,*p<0.05 vs. normal group). **B:** *O*-GlcNAc/OGA ratio in granulocytes (n=51). **C:** Lymphocyte *O*-GlcNAc/OGA ratio (n=52, #p<0.05). Values are expressed as mean ± SEM.

3.9.3 Leukocyte *O*-GlcNAc/OGA ratio increases with rising HbA1c levels:

For total leukocyte sample we recorded a highly significant elevation (68.5 ± 5%) in the *O*-GlcNAc/OGA ratio between normal and diabetic samples (Figure 3.16A). Additionally, differences were detected between normal and pre-diabetic individuals (42.7 ± 8.6%) ($p<0.05$) as well as between pre-diabetic and diabetic subjects (18.1 ± 5.6%) ($p<0.05$) (Figure 3.16A). Granulocyte *O*-GlcNAc/OGA ratios showed no significant differences even though slight increases were observed (Figure 3.16B). However, lymphocytes displayed significant increases in *O*-GlcNAc/OGA ratios in diabetic individuals (25.4 ± 5.9%) compared to pre-diabetic participants (Figure 3.16C).

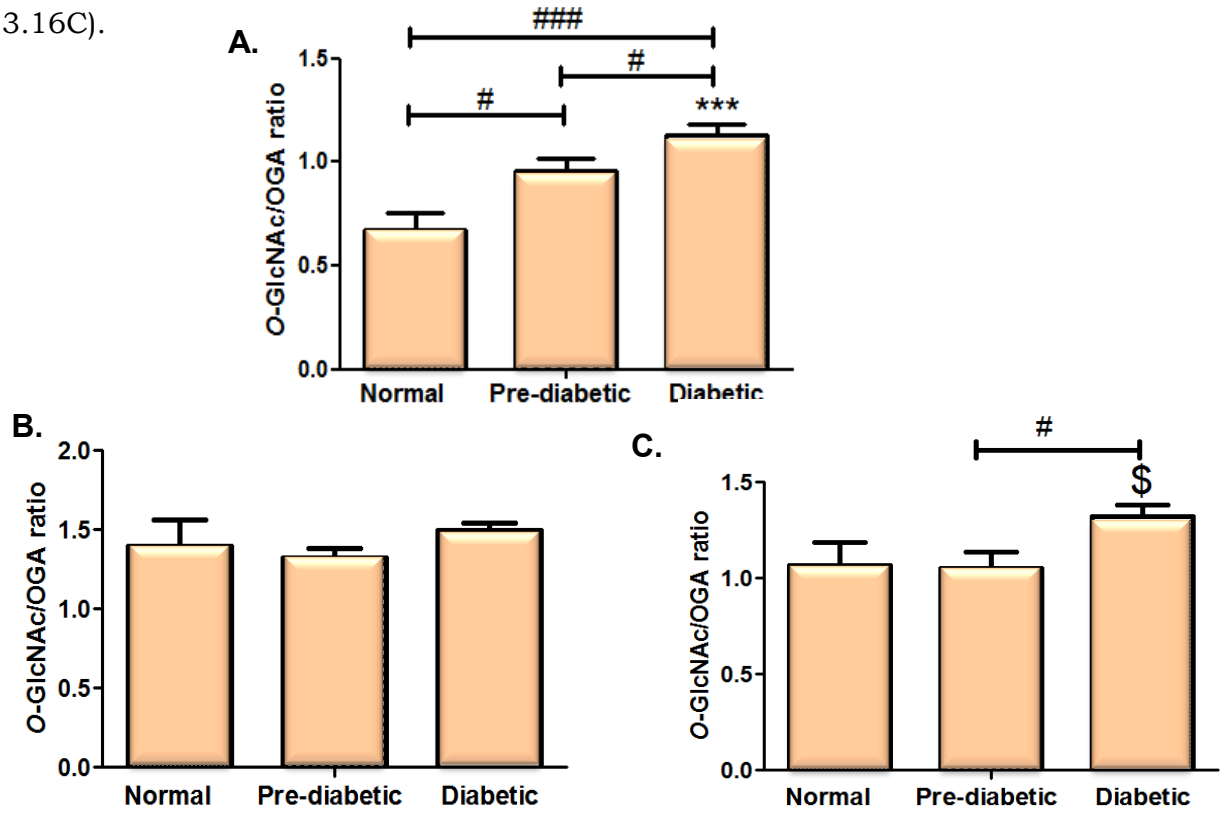


Figure 3.16 Increased *O*-GlcNAc/OGA ratio in leukocytes when characterized according to HbA1c levels (flow cytometry).

A: *O*-GlcNAc/OGA ratio in total WBC population- prediabetic and diabetic subjects versus healthy individuals (n=50, $^{###}p<0.001$, $^{\#}p<0.05$, $^{***}p<0.001$ vs. normal group).**B:** Granulocyte *O*-GlcNAc/OGA ratio levels (n=49, no significant differences observed) **C:** Lymphocyte *O*-GlcNAc/OGA ratio's (n=49, $^{\#}p<0.05$, $^{\$}p<0.05$ vs. pre-diabetic group). Values are expressed as mean ± SEM.

3.10 Discrepancies between diagnostic tests and defining criteria

An important aspect in the field of diabetes diagnosis, and thus for our study, are the current incongruities between existing diagnostic tools and organization-specific criteria (as discussed in the Introduction section 1.3.2). We therefore decided to evaluate the severity of these discrepancies in our study population.

3.10.1 Discrepancies between FPG and HbA1c

The FPG criteria for defining diabetes, both WHO and ADA, is >7 mmol/L. Likewise, an HbA1c of >6.5% is diagnosed as diabetes (refer to Table 1.3). After carefully examination of our data, we found that 13.6% of our study population was either >7 mmol/L, <6.5% or <7 mmol/L, >6.5%.

Next, the levels of *O*-GlcNAcylation in these two groups were evaluated, as well as for the other possibilities, i.e. <7 mmol/L, <6.5% and >7 mmol/L, > 6.5%. The results indicated a significant increase between <7 mmol/L, <6.5% and >7 mmol/L, <6.5%. Also, significant differences were observed between >7mmol/L, <6.5% and <7 mmol/L, >6.5%; >7 mmol/L, <6.5% and >7 mmol/L, > 6.5%, along with <7 mmol/L, <6.5% and >7 mmol/L, > 6.5% (Figure 3.17).

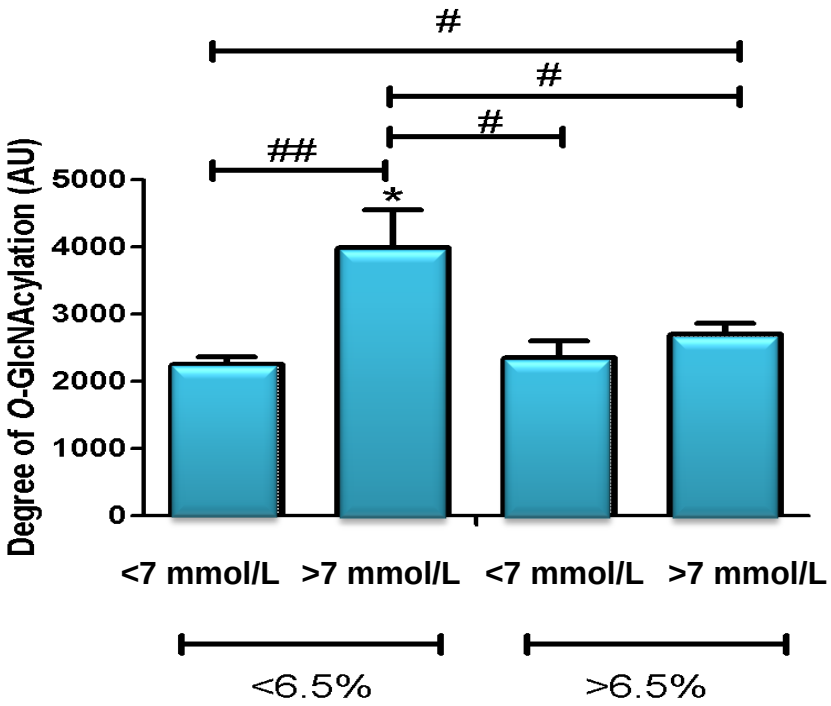


Figure 3.17 Differences in *O*-GlcNAcylation between groups established from discrepancies between HbA1c and FPG.

(n=73, ##p<0.01, #p<0.05,*p<0.05 vs. <7 mmol/L, <6.5%. AU: Arbitrary units. Values are expressed as mean ± SEM.

3.10.2 Discrepancies between WHO and ADA

As discussed, the WHO and ADA both define diabetes with an FPG >7.0 mmol/L. However, their respective normal and pre-diabetic classifications result in a specific range of glucose concentrations (5.6-6.0 mmol/L). This results in dissimilar characterization, i.e. individuals that fall within this glucose range will be classified as "normal" according to WHO, but "pre-diabetic" according to ADA guidelines. We investigated our data and found that 12.9% of our study population fell into this glucose range.

We next investigated *O*-GlcNAc modified protein levels in the 5.6-6.0 mmol/L group by comparison to the ADA normal (<5.6 mmol/L), WHO normal (<6.1 mmol/L), ADA pre-diabetic (5.6-6.9 mmol/L) and WHO pre-diabetic (6.1-6.9 mmol/L) groups. Here we identified significant differences between ADA normal and pre-diabetic groups, and WHO normal and pre-diabetic groups. Interestingly, *O*-GlcNAcylation

could distinguish between the ADA normal group and WHO pre-diabetic group, as well as between the WHO pre-diabetic group and the 5-6-6.1 mmol/L group (Figure 3.18).

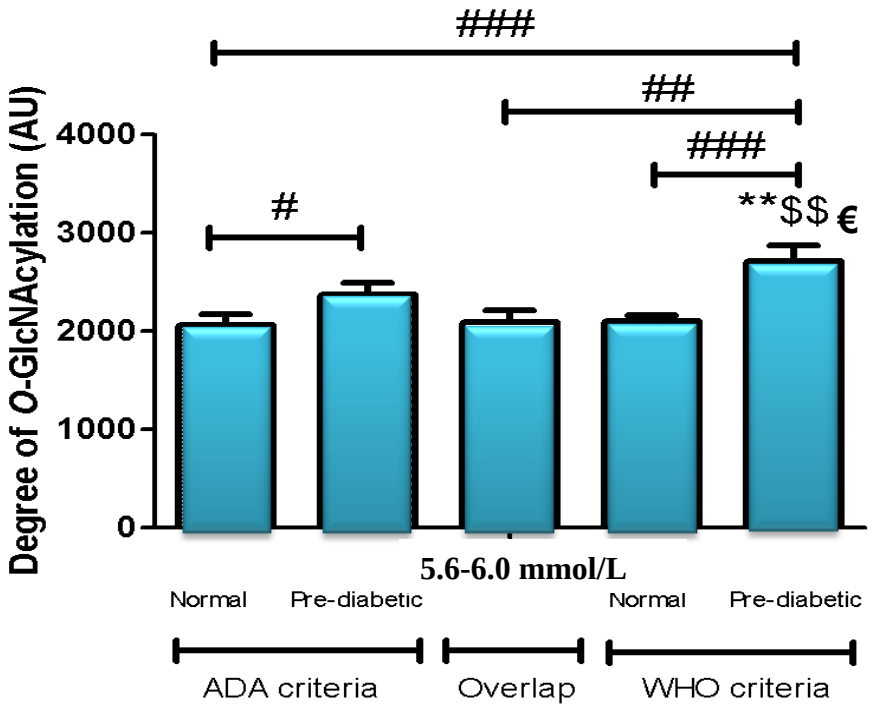


Figure 3.18 Differences in O-GlcNAcylation between groups established from discrepancies between WHO and ADA diagnostic criteria.

(n=62, ###p<0.01, ##p<0.01, #p<0.05,**p<0.01 vs. ADA normal), $$p<0.01 vs. (WHO pre-diabetic), €p, 0.05 vs 5-6-6.0 mmol/L. AU: Arbitrary units. Values are expressed as mean ± SEM.

3.11 Characterization of study population

We next examined whether the above inconsistencies had any effect on the characterization of our recruited subjects. When subjects were characterized according to ADA criteria, almost a third of the population were in the normal group, with 46.8% of individuals characterized as diabetic and 22.1% as pre-diabetic (Figure 3.19A). When WHO guidelines were implemented to characterize individuals, the diabetic group understandably remained the same, but the normal group increased to 41.5% of the population, leaving only 11.7% of individuals characterized as pre-diabetic (Figure 3.19B). By contrast, characterization

according to HbA1c levels decreased the normal group to a small 10.9% of the study population, with the diabetic group making up more than half of the individuals (56.7%), and the pre-diabetic group comprising 31.5% of subjects (Figure 3.19C).

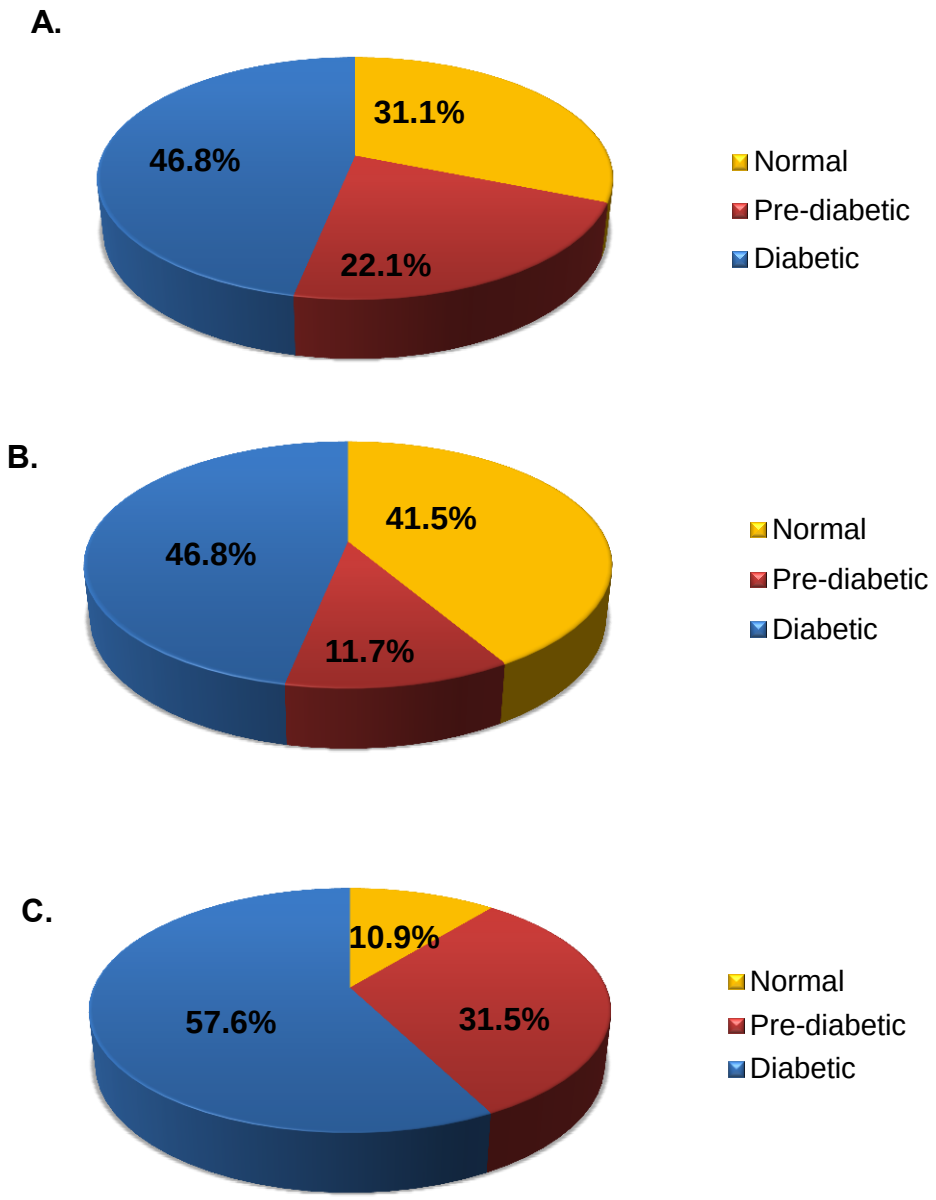


Figure 3.19 Percentage of population characterized as normal, pre-diabetic and diabetic according to A: ADA criteria B: WHO guidelines C: HbA1c levels.

3.12 "True" normal, pre-diabetic and diabetic individuals

The significant differences between ADA, WHO and HbA1c in the characterization above (section 3.11) lead us to determine how many individuals are "true" normal, pre-diabetics and diabetics. In other words we ascertained how many individuals are characterized as normal, pre-diabetic or diabetic by *all* three guidelines.

We discovered that 10% of the population was normal according to all three guidelines (<5.6 mmol/L; <5.7%), 8.75% were true pre-diabetics (6.1-7 mmol/L; 5.7-6.5%), and 42.5% of recruited subjects were true diabetics (>7.0 mmol/L; > 6.5%). This means that 38.75% of the study participants may be categorized into alternative groups, i.e. dependent on which criteria are implemented (grey section in pie chart) (Figure 3.20).

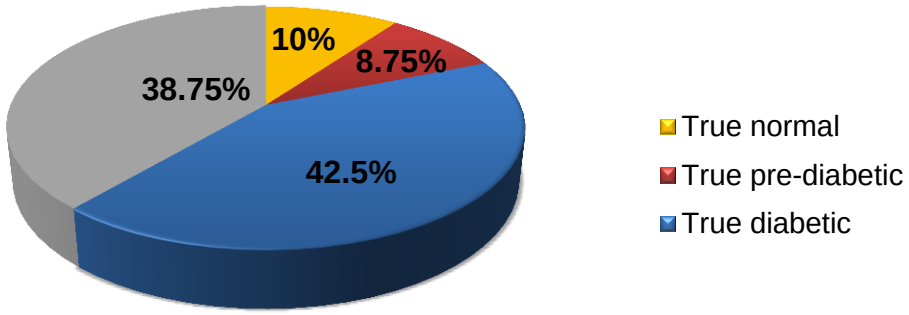


Figure 3.20 Percentage of population characterized as normal, pre-diabetic and diabetic according to all three criteria (ADA, WHO, HbA1c).

3.12.1 The investigation of *O*-GlcNAcylation between "true" normal, pre-diabetic and diabetic individuals

We subsequently determined the flow-cytometric *O*-GlcNAc levels for the "true" normal, pre-diabetic and diabetic individuals and no significant differences were found in the total leukocyte population (Figure 3.21A). However, for both the granulocytes and lymphocytes there were significant increases of 36.7 ± 5.9% and 63.6 ± 8.5%, respectively, between normal and diabetic individuals (Figure 3.21B and Figure 3.21C).

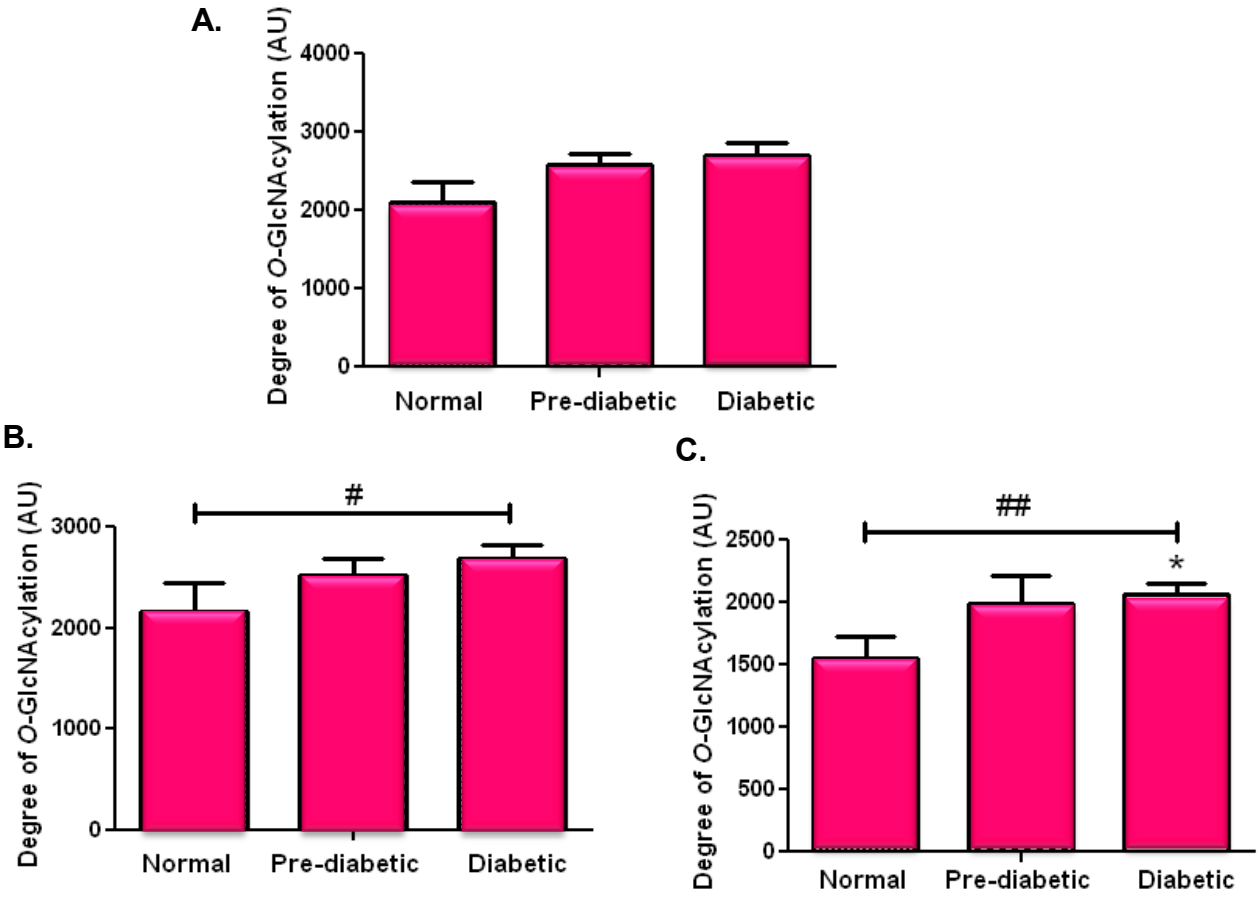
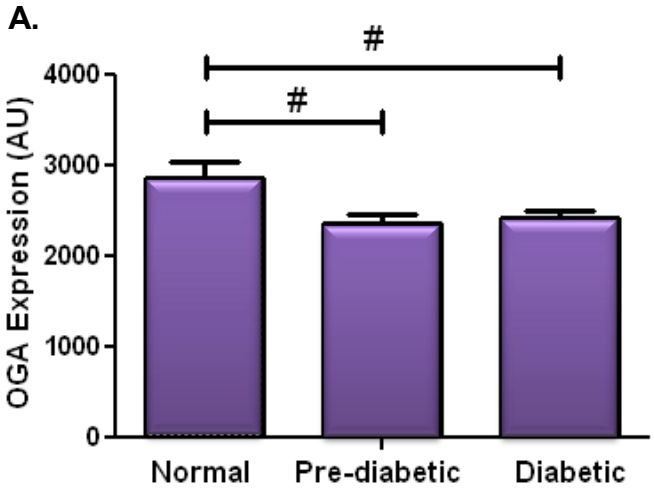


Figure 3.21 Increased *O*-GlcNAcylation in leukocytes of "true" diabetic individuals.

A: *O*-GlcNAcylation in total WBC population (n=49) **B:** Granulocyte *O*-GlcNAcylation levels (n=37, $^{\#}p<0.05$) **C:** Lymphocyte *O*-GlcNAc protein levels (n=37, $^{\#\#}p<0.01$, $^{*}p<0.05$ vs. normal group). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.12.2 Analyzing OGA expression between "true" normal, pre-diabetic and diabetic individuals

OGA expression amongst these individuals yielded more sensitive results. In the total leukocyte population there were significant differences detected between normal and pre-diabetic individuals (decrease of 17.9 ± 3.5%), as well as between normal and diabetic subjects (decrease of 15.7 ± 3%) (Figure 3.22A). By contrast, OGA expression was significantly increased in the granulocytes of diabetics compared to normal subjects (Figure 3.22B). Moreover, for lymphocytes increases of 8.7 ± 7.1% ($p<0.05$) and 16.2 ± 2.6% ($p<0.01$) were found in the pre-diabetic and diabetic groups, respectively (Figure 3.22C).



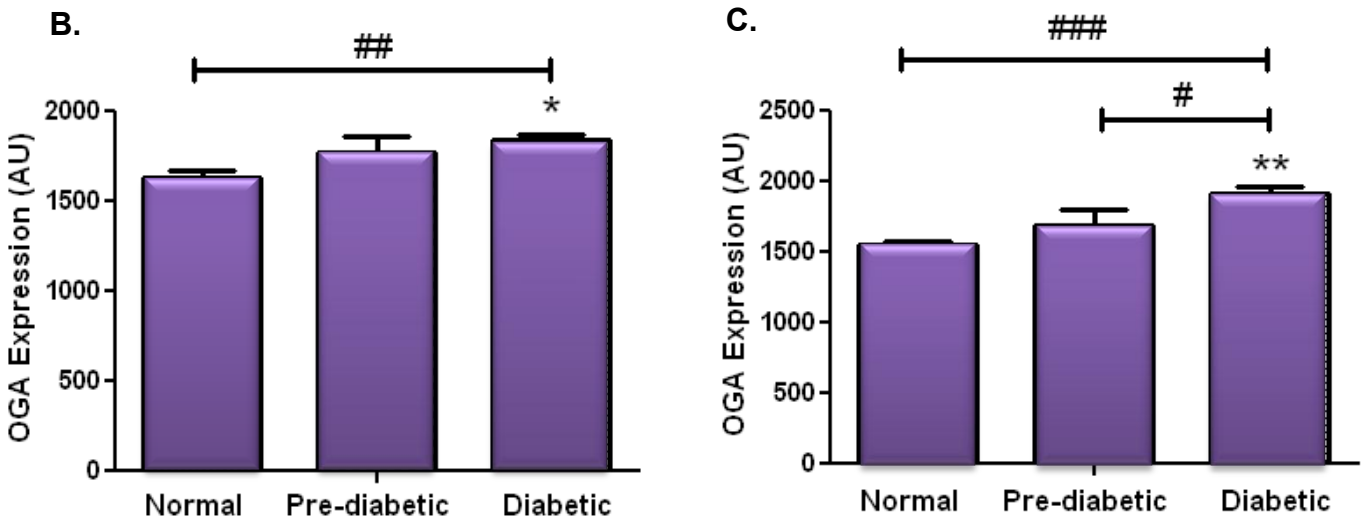
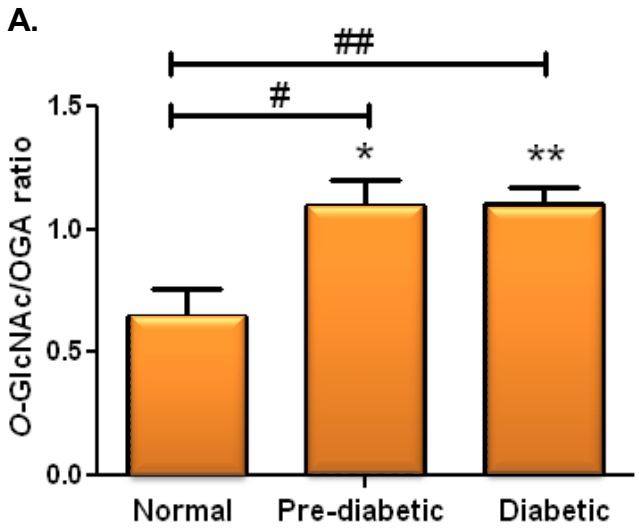


Figure 3.22 OGA protein expression in leukocytes of "true" diabetic individuals.

A: Total leukocyte population's OGA expression (n=31, #p<0.05) **B:** Granulocyte OGA protein levels (n=34,##p<0.01, *p<0.05 vs. normal group) **C:** OGA expression in the lymphocytes(n=35, ###p<0.001, #p<0.05, **p<0.01 vs. normal group). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.12.3 Determining *O*-GlcNAc/OGA ratios for "true" normal, pre-diabetic and diabetic participants

The *O*-GlcNAc/OGA ratios distinguish between normal and pre-diabetic and between normal and diabetic individuals. Here our data show a significant increase in the pre-diabetic (68.5 ± 1.6%) and diabetic (70.3 ± 9.7%) groups compared to normal subjects (Figure 3.23A). However, no differences were detected for granulocytes or lymphocytes (Figure 3.23B and Figure 3.23C).



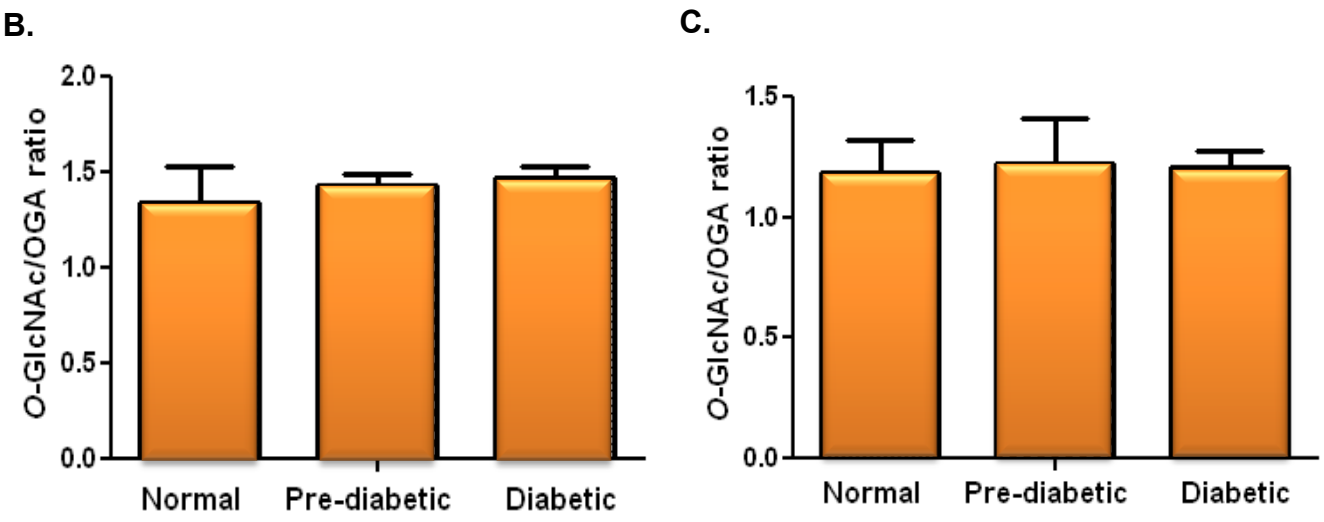


Figure 3.23 *O*-GlcNAc/OGA ratio evaluated in leukocytes of "true" diabetic individuals.

A: *O*-GlcNAc/OGA ratio in total WBC population (n=31, ##p<0.01, #p<0.05, **p<0.01 *p<0.05 vs. normal group) **B:** Granulocyte *O*-GlcNAc/OGA levels (n=34) **C:** Lymphocyte *O*-GlcNAc/OGA ratio's (n=35). AU: Arbitrary units. Values are expressed as mean ± SEM.

3.13 The inaccuracy of FPG (case study)

Due to our study's reliance on FPG, a specific case study must be noted: two, very healthy and fit male subjects were specifically recruited with the intention of obtaining valuable control data. The analysis of *O*-GlcNAcylation took place blindly and we did not have access to FPG values until after *O*-GlcNAcylation levels were finalized. As expected, both men presented with relatively low *O*-GlcNAc levels in terms of other study recruits. However, the test results confirmed FPG levels of 6.3 and 6.4 mmol/L, thereby characterizing both men as pre-diabetic (according to both WHO and ADA standards). Due to the health conscious nature of both individuals, five year FPG records were available for us to scrutinize. Here we found that the most recent reading (performed less than a month prior to our reading) showed that the individuals presented with FPG values of 5.4 and 4.3 mmol/L. This translates into a discrepancy of 0.9 and 2.1 mmol/L, respectively. After averaging the five year record for each individual, we calculated that their FPG's

were 5.4 and 5.1 mmol/L, respectively. Our calculations confirmed that if these two recruits were included in the pre-diabetic group, then their lower *O*-GlcNAc levels would have skewed the mean *O*-GlcNAcylation reading, and resulted in a smaller difference between normal and pre-diabetic groups.

3.14 Investigation into the combination of insulin and fasting plasma glucose levels

Individuals in our study were recruited through two sources, Stellenbosch and an existing Bellville-South diabetic study. The latter had the resources to measure the participants' insulin levels, and therefore we gained access to this data for a subset of our recruited individuals (n=55). We decided to investigate the insulin levels in combination with the glucose levels, and characterize our population according to glucose/insulin groups. We retained our three groups for FPG: normal, pre-diabetic and diabetic (ADA criteria was used in order to include more individuals in the pre-diabetic group), and we also established two insulin groups. The healthy insulin reference range is between 2.1 and 10 μU/mL, and therefore we included a normal insulin (2.1 and 10 μU/mL) and a high insulin (>10 μU/mL) group (a low insulin group was not necessary since no individuals presented with insulin values < 2.1 μU/mL). When combined, these two variables resulted in a total of six groups: Normal glucose normal insulin (NGNI); Pre-diabetic glucose normal insulin (PGNI); Normal glucose high insulin (NGHI); Pre-diabetic glucose high insulin (PGHI); High glucose high insulin (HGHI); High glucose normal insulin (HGNI).

3.14.1 Characterization into glucose/insulin groups

10.9% of individuals presented with NGNI while a similar number (9.1%) were characterized with PGNI and NGHI. Moreover, 14.5% fell into the PGHI group while the majority of participants were classed into the HGHI category (40%). Finally, 16.4% of the study population presented with HGNI (refer to Figure 3.24)

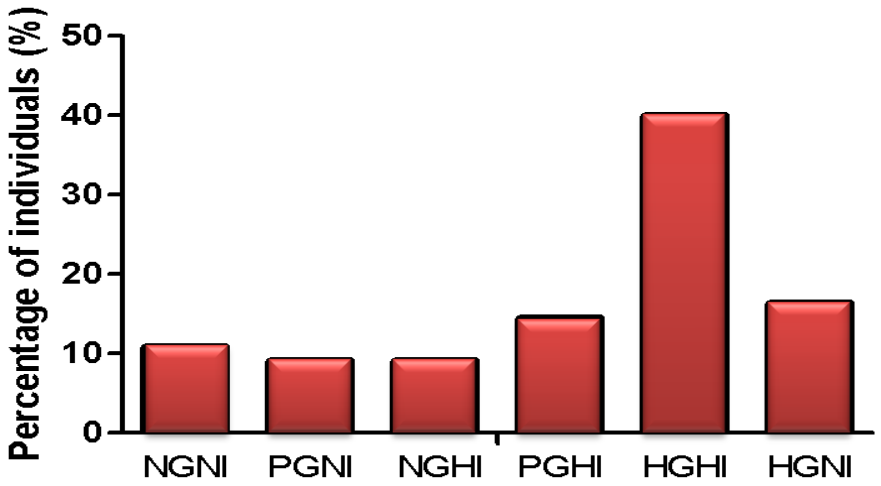


Figure 3.24 Percentage of Bellville-South population characterized according to glucose/insulin groups. NGNI: Normal glucose normal insulin; PGNI: Pre-diabetic glucose normal insulin; NGHI: Normal glucose high insulin; PGHI: Pre-diabetic glucose high insulin; HGHI: High glucose high insulin; HGNI: High glucose normal insulin (n=55).

3.14.2 *O*-GlcNAcylation and *O*-GlcNAc/OGA ratio differs between glucose/insulin groups

We next evaluated *O*-GlcNAcylation levels for each glucose/insulin group. Here we observed a significant decrease in *O*-GlcNAc modified leukocyte proteins in NGHI compared to NGNI (-37.6 ± 9.3%), while significant increases of 54.1 ± 12.9% and 80.2 ± 12.1% were detected in HGHI and HGNI, respectively (versus NGHI) (Figure 3.25A).

We also investigated the *O*-GlcNAc/OGA ratios between the different groups and found a similar pattern. The *O*-GlcNAc/OGA ratio decreased by 61.1 ± 14.3% in the NGHI group versus the PGNI category, while significant increases were detected in HGHI and HGNI, respectively (versus NGHI) (Figure 3.25B).

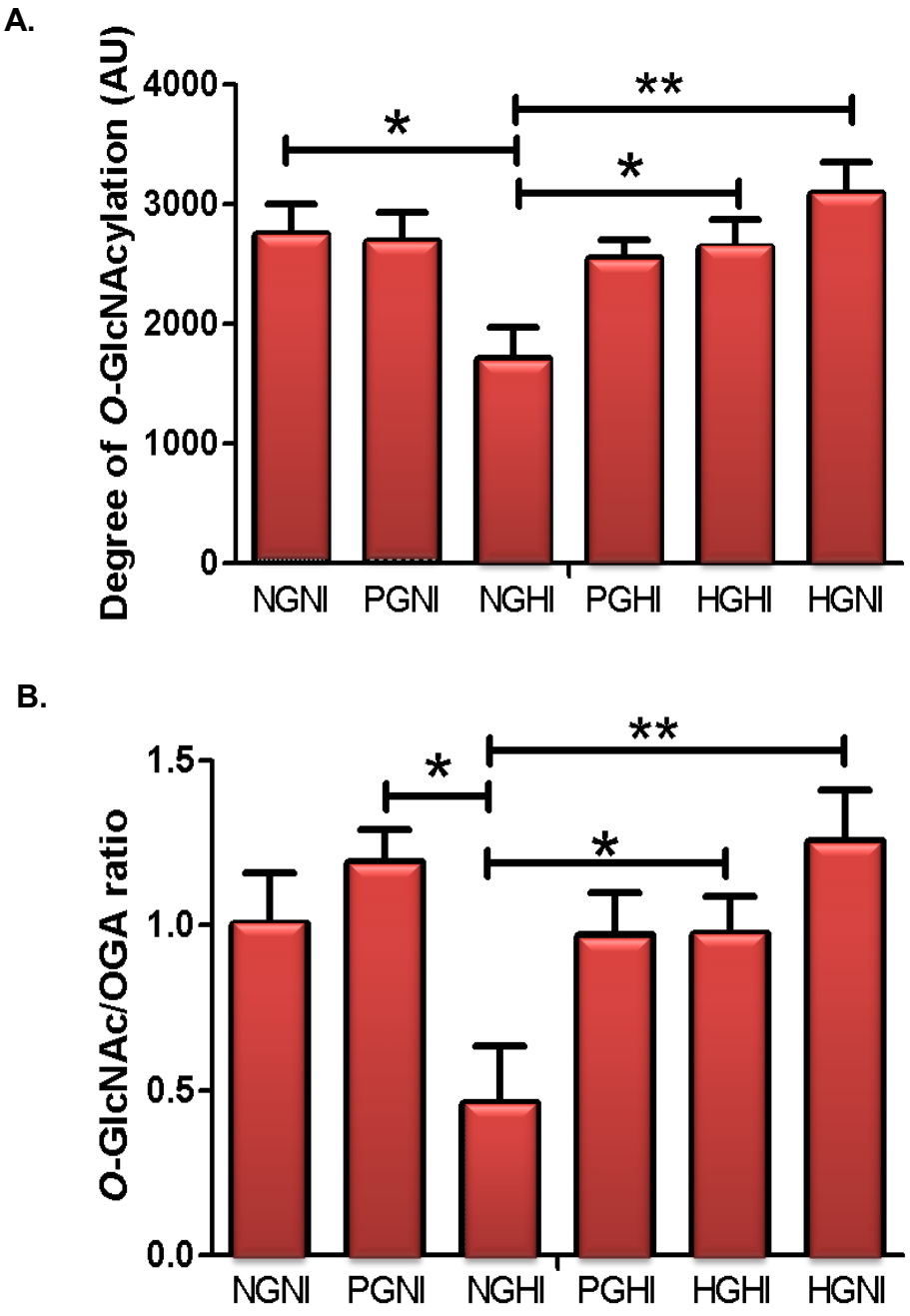


Figure 3.25 *O*-GlcNAcylation levels and *O*-GlcNAc/OGA ratios compared to glucose/insulin groups.

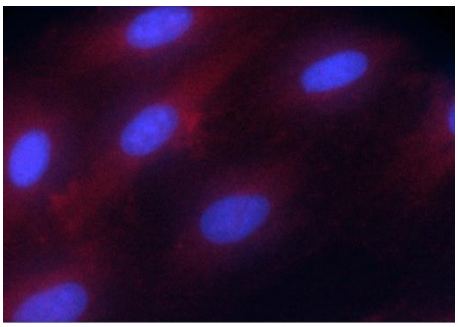
A: Degree of *O*-GlcNAcyaltion versus various glucose/insulin groups (n=55, **p<0.01, *p<0.05) AU: Arbitrary units. **B:** *O*-GlcNAc/OGA ratio (n=55, **p<0.01, *p<0.05). Values are expressed as mean ± SEM.

3.15 Determining the effect of insulin on HBP flux

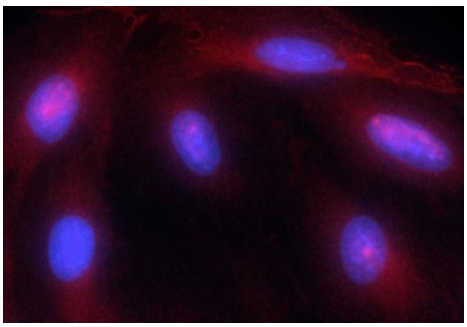
Due to the interesting findings in section 3.12, we decided to evaluate the effect of three different doses of insulin (0.1 µM, 1 µM, 10 µM) on *O*-GlcNAc modified protein levels under high (25 mM) and low glucose (5.5 mM) culturing conditions (H9c2 rat cardiomyoblast cell line). We performed the analysis using immunofluorescence microscopy and Western blotting.

3.15.1 Immunofluorescence microscopy

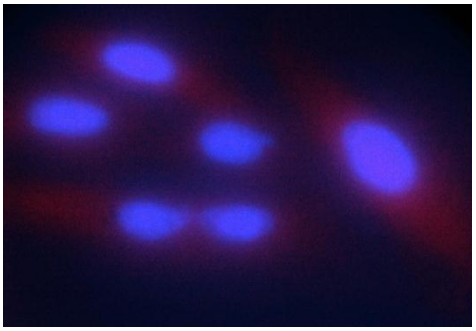
Fluorescence microscopy was used as a qualitative technique. Under low glucose (LG) culturing conditions, the only visible difference in *O*-GlcNAcylation is a distinct decrease in the high insulin group (10 µM) (Figure 3.26).



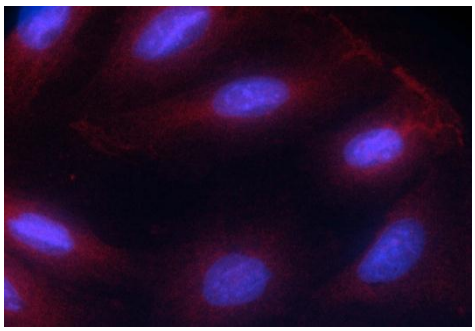
Dye control



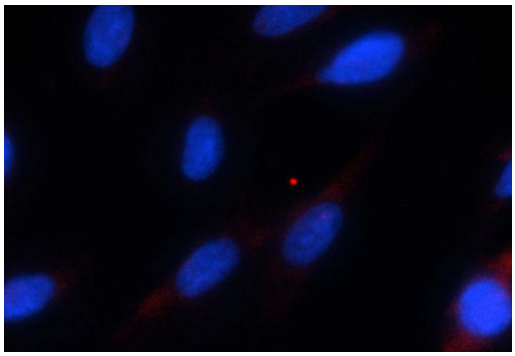
Basal control



0.1 µM Insulin



1 µM Insulin

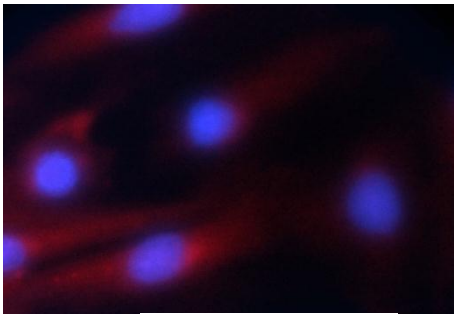


10 µM Insulin

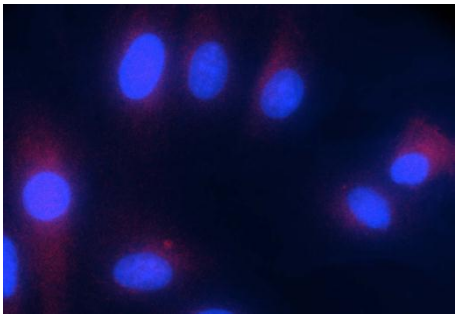
Figure 3.26 Effect of insulin on *O*-GlcNAcylation under low glucose culturing conditions (5.5 mM) in H9c2 cells (immunofluorescence microscopy).

Representative images from immunohistochemistry (qualitative). H9c2s were stained for *O*-GlcNAc (red) and Hoechst dye (blue). **A:** Dye control **B:** Basal control **C:** 0.1 µM insulin treatment **D:** 1 µM insulin treatment **E:** 10 µM insulin treatment.

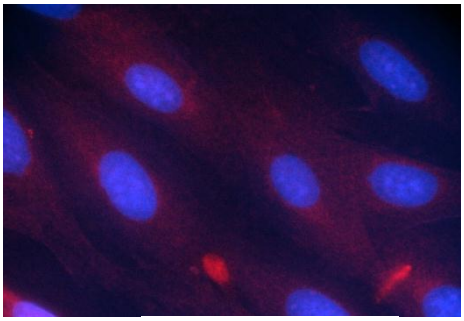
An identical pattern was observed for cells cultured under high glucose conditions, with the only noticeable difference being reduced *O*-GlcNAc fluorescent signal in the high insulin group (10 µM) (Figure 3.27).



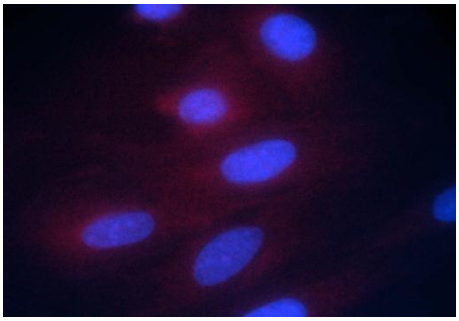
Dye control



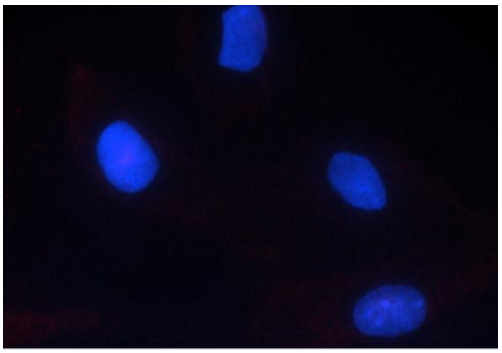
Basal control



0.1 µM Insulin



1 µM Insulin



10 µM Insulin

Figure 3.27 Effect of insulin on *O*-GlcNAcylation under high glucose conditions (25 mM) in H9c2 cells (immunofluorescence microscopy).

Representative images from immunohistochemistry (qualitative). H9c2s were stained for *O*-GlcNAc (red) and Hoechst dye (blue). **A:** Dye control **B:** Basal control **C:** 0.1 µM insulin treatment **D:** 1 µM insulin treatment **E:** 10 µM insulin treatment.

3.15.2 Western blotting

We next employed Western blotting analysis as a quantitative technique to investigate changes in *O*-GlcNAcylation in response to varying insulin levels (Figure 3.28A). We compared high glucose and low glucose cells in the following groups: LGC: Low glucose control; LGLI: Low glucose low insulin; LGMI, Low glucose medium insulin; LGHI: Low glucose high insulin; LGPC: Low glucose positive control; HGC: High glucose control; HGLI: High glucose low insulin; HGMI: High glucose medium insulin; HGHI: High glucose high insulin; HGPC: High glucose positive control (refer to materials and methods section 2.7) No significant differences in *O*-GlcNAcylation were detected between any of the groups (Figure 3.28B).

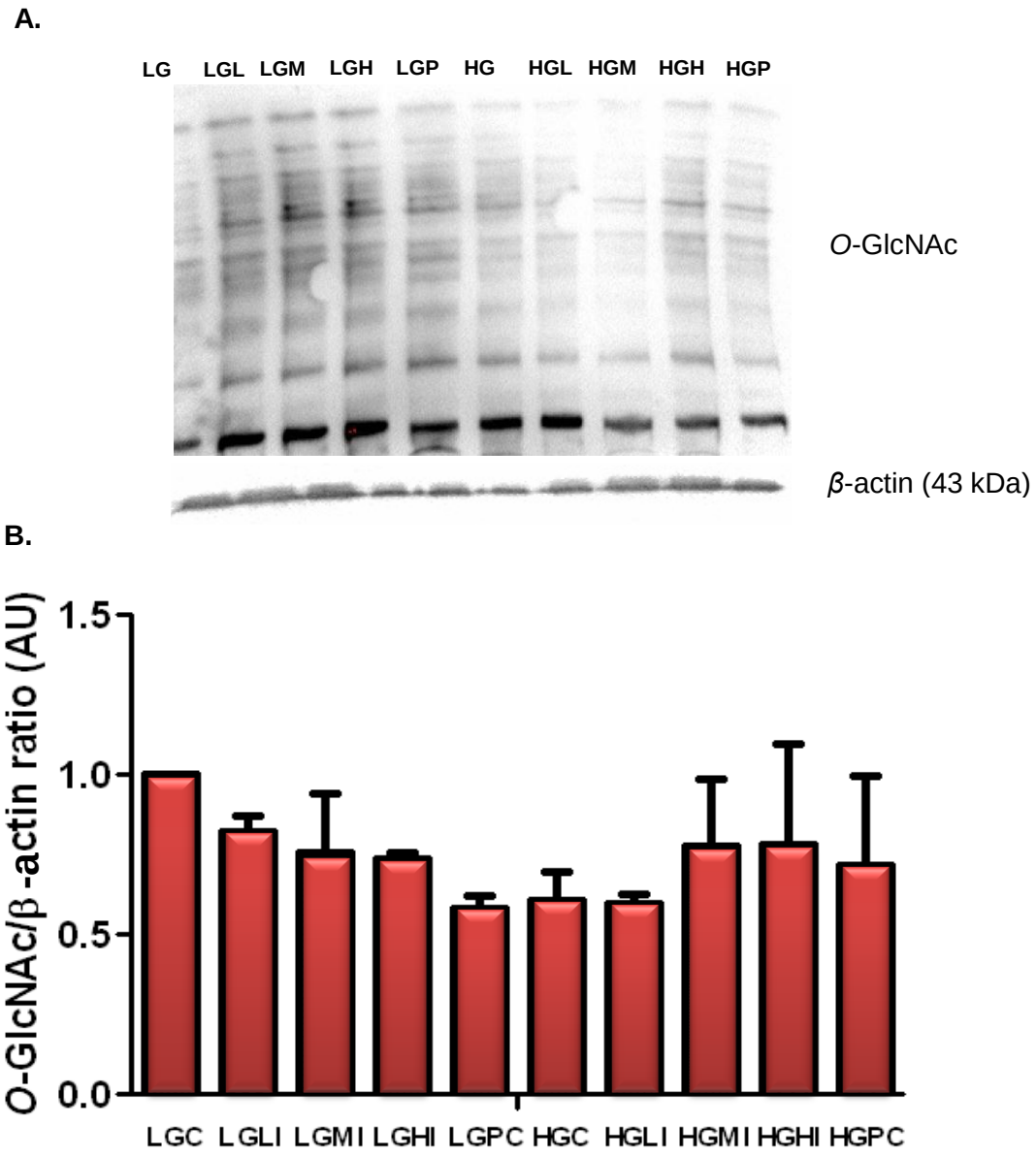


Figure 3.28 Western blot analysis of *O*-GlcNAcylation in insulin-treated H9c2 cells cultured under high and low-glucose conditions.

A: Representative *O*-GlcNAc blot **B:** Bar graphs show volumetric quantification of *O*-GlcNAc levels compared to low glucose control (LGC) group (normalized to β-actin) (n=3). AU: Arbitrary units. Values are expressed as mean ± SEM.

4.

Discussion

Type 2 diabetes remains under-diagnosed despite the availability of several diagnostic tests [32]. There are several obstacles that impede successful detection of diabetes. These include various limitations of the current diagnostic assays (section 1.33), incongruities between alternative diagnostic approaches and significant variation between organization-specific characterization criteria (section 1.3.2). Due to the significant vulnerability of diabetic patients to a plethora of cardiovascular and non-vascular diseases [29], we propose that the augmented detection of diabetes, and its precursor, pre-diabetes, would allow for: a) delayed progression of these complications, b) earlier and more extensive treatment opportunities, c) improved prognostic outcomes and d) the enhanced focus on successful preventative strategies. Together this provides impetus for the development of novel prognostic assays subject to fewer complexities. For example, hyperglycemia-induced HBP flux is linked to insulin resistance and associated with metabolic defects responsible for the progression of diabetes (reviewed in [188, 192]). Since *O*-GlcNAcylation of target proteins is subject to intracellular glucose availability (discussed in [193]), we hypothesized that the extent of *O*-GlcNAcylation is a useful tool to assess glucose metabolism of individuals with both subtle and overt glucose dysregulation, thereby representing a novel marker for the increased efficiency and earlier diagnosis of diabetes.

The main findings of this study are: 1) Differential *O*-GlcNAc modification between diverse leukocyte sub-populations; 2) Pre- and diabetic individuals display increased leukocyte *O*-GlcNAcylation; 3) Differential leukocyte OGA

expression found in diabetic subjects; 4) OGT expression did not significantly change for any of the study recruits investigated 5) Elevated *O*-GlcNAc/OGA ratios in pre-diabetic and diabetic individuals.

4.1 Differential *O*-GlcNAc modification between diverse leukocyte sub-populations

The major principle of flow cytometry (our fundamental technique) is its ability to determine the properties of individual particles in solution [245]. The forward and side scatter channels separate cells according to size and granular content, respectively [243]. Moreover, CD45 is an antigen present in all WBCs but expressed in varying magnitude between separate leukocyte lineages [244]. Our data confirm that the combination of flow cytometric light scattering properties and simultaneous quantification of CD45 surface antigen expression can be used for the accurate identification of leukocyte sub-populations [242] (Figure 3.1).

Due to the accurate differentiation of leukocyte sub-populations, we initially compared the degree of *O*-GlcNAcylation in lymphocytes compared to granulocytes. Interestingly, granulocytes exhibited markedly higher levels of *O*-GlcNAc modified proteins compared to lymphocytes (Figure 3.2). This, to our knowledge, is a unique observation and we found no previous literature comparing *O*-GlcNAcylation in leukocyte sub-populations. Moreover, OGA expression was essentially identical in the granulocytes and lymphocytes (Figure 3.3), thereby adding to the perplexity of this finding. What is the importance of the disparity in *O*-GlcNAc modification between granulocytes and lymphocytes? Since no differences in OGA expression occurred (Figure 3.3), this remains unclear, but investigation into their respective

roles within the immune system allows us to propose possible explanations for these unique results.

An immediate thesis why neutrophils displayed greater *O*-GlcNAcylation may relate to research showing that *O*-GlcNAc plays a significant role in the efficient execution of the neutrophil's response towards infection [246]. Previous research work provided evidence that neutrophil motility and signaling also depend on *O*-GlcNAc modification [240]. However, further investigation uncovered that *O*-GlcNAcylation also plays a central role in the regulation and activation of T and B lymphocytes [247]. These data therefore support the notion that *O*-GlcNAcylation is actively involved in the proper functioning of neutrophils and lymphocytes. But to what extent does it play a role? Diverse proteins are present within the lymphocytes and neutrophils [248, 249], and it is likely that neutrophil proteins not shared with lymphocytes are potential *O*-GlcNAc targets. Moreover, since several proteins present in both sub-populations are expressed in varying amounts [250] there is a strong possibility that neutrophils perhaps have a larger quantity of specific *O*-GlcNAc modified proteins.

Another factor to consider here is that *O*-GlcNAc cycles (as previously mentioned) [190]. More importantly, *O*-GlcNAc cycling occurs at different rates for various proteins [87] and hence such variation may also result in the differences we observed between granulocytes and lymphocytes. Finally, previous research work found that neutrophils displayed higher levels of oxidative stress compared to lymphocytes [251]. Since oxidative stress is responsible for HBP activation [142](refer Introduction section 1.6), it is likely that varying *O*-GlcNAcylation found is simply the direct result of differential ROS production between lymphocytes and neutrophils.

Although significant advances have been made in the development of specific and sensitive methods to quantify the extent and identity of specific *O*-GlcNAcylation sites [252, 253], these techniques are only in their infancy. This is therefore a major reason for the limited progress to fully elucidate the functional role(s) of *O*-GlcNAc [246]. We therefore propose that determination of site-specific *O*-GlcNAc modification in neutrophils and lymphocytes is vital in clarifying its differential response, and also in providing firm evidence to support our proposals made here.

O-GlcNAc moieties are dynamically attached and removed from target proteins under the robust control of two conserved enzymes, OGT and OGA, respectively [230, 254]. In order to fully investigate the diagnostic value of O-GlcNAcylation, we subsequently explored the degree of O-GlcNAc modified proteins and OGA/OGT expression in sub- and total leukocyte populations of recruited individuals. With this broad scope, we set out to further advance our understanding of O-GlcNAcylation in pre- and overt diabetic individuals, with the eventual aim to offer diagnostic utility. For this study normal, pre-diabetic and diabetic individuals were distinguished with both ADA and WHO FPG defining criteria, as well as the ADA's recommended HbA1c levels.

4.2 Pre- and diabetic individuals display increased leukocyte *O*-GlcNAcylation

Hyperglycemia increases *O*-GlcNAcylation through increased HBP flux [192, 221]. Indeed, several cell-based and animal studies showed increased *O*-GlcNAcylation in response to augmented glucose levels [168, 209, 215, 221, 255-257]. We therefore proposed a "translation" of this response into the clinical setting and hypothesized increased *O*-GlcNAc modified protein levels in pre-diabetic and diabetic individuals.

Differences between ADA, WHO and HbA1c characterizations were subtle. However, for all three categories *O*-GlcNAcylation increased with higher glucose and HbA1c levels (Figure 3.4-3.6).

For the total leukocyte population, diabetic individuals exhibited increased leukocyte *O*-GlcNAcylation (characterized according to ADA fasting glucose and HbA1c levels), but could not distinguish between pre- and overt diabetic subjects (Figure 3.4A and 3.6A). However, *O*-GlcNAc-modified protein levels increased in diabetic and pre-diabetic individuals versus the normal group (WHO criteria) (Figure 3.5A). These data clearly illustrate how the sensitivity of *O*-GlcNAc is affected when participants are alternatively characterized, thereby highlighting the repercussions of discordant diagnostic threshold values. Since the WHO pre-diabetic recommendations only include individuals with a FPG of >6.1mmol/L, *O*-GlcNAcylation of pre-diabetic groups is in a higher range compared to the ADA, emphasizing its ability to distinguish between normal and pre-diabetic individuals.

The leukocyte sub-typing further strengthened the total WBC population data and provided additional insights in this regard, i.e. granulocyte *O*-GlcNAcylation (ADA and WHO) differentiated between normal and diabetic individuals more significantly than in the total leukocyte population. More importantly, the vital distinction between normal and pre-diabetic subjects was also achieved (Figure 3.4B-3.5B). Furthermore, when recruits were characterized according to ADA FPG and HbA1c levels, the degree of lymphocyte *O*-GlcNAcylation allowed for differentiation between healthy and diabetic subjects, and also pre-diabetic and diabetic individuals (Figure 3.4C and 3.6C).

We found that differences within the leukocyte sub-type data are statistically more robust versus the total leukocyte population. It is reasonable to postulate that such variations in sensitivity are due to differences in gating accuracy (flow cytometry). Since both lymphocyte and granulocyte populations can be gated and therefore defined with much higher precision compared to the total leukocyte sample, this results in greater reproducibility. By contrast, the total leukocyte population may include erythrocytes and other debris, potentially making gating more variable between samples and thereby decreasing sensitivity.

Nevertheless, these results strongly validate the highly sensitive nature of *O*-GlcNAc modification in response to nutrients and cellular stress (hyperglycemia). Collectively *O*-GlcNAc-modified protein levels were, although not concurrently, able to differentiate between normal and pre-diabetic, normal and diabetic, and pre-diabetic and diabetic individuals.

Although flow cytometry was employed as our principal technique, *O*-GlcNAcylation was additionally investigated with immunofluorescence microscopy and Western blotting (Materials and methods section 2.5.2 and 2.5.3). We found a significant increase in *O*-GlcNAcylation in diabetic individuals (ADA criteria) when employing microscopy (Figure 3.4E). However, increases observed for the other categories (WHO and HbA1c) were not statistically significant (Figure 3.5E and 3.6E). Several problems were experienced when executing immunofluorescence microscopy that could justify the large variation and therefore the loss of statistical power.

Troubleshooting formed a large part of this technique, and as a result, very few suitable images were available for quantification. The two major problems we experienced were: a) the fixation of cells, and b) the lack of adherence of leukocytes

to the chambers. Firstly, upon microscopy analysis we found that cell morphology was completely distorted. Further optimization uncovered the fixative agent as the culprit, and as a result we employed 6% formaldehyde/PBS solution as a substitute to the 1:1 methanol/acetone fixative (commonly used in alternative protocols). In support, a study performed by Hoetalmans *et al.* (2001) showed the complete loss of intracellular integrity when using methanol:acetone versus formaldehyde [258]. Our modified approach resulted in the conservation of leukocyte morphology and a significant improvement in image quality.

Secondly, the lack of adherence and subsequent loss of cells resulted in a poor representation of the total leukocyte population. As a result of the small quantity of adhering cells, the majority of the images could only be used as a qualitative measure of *O*-GlcNAcylation. Here we typically analyzed 50-100 cells (per sample) for quantification versus 10, 000 cells by flow cytometry. This therefore clearly highlights the disparity in quantitative power between these two techniques. Nevertheless, despite these difficulties, subtle differences were still detected and the qualitative images yielded visible differences (representative of several images) in support of our flow cytometric data (Figure 3.4-3.6F).

No distinctions were made between alternative threshold values or leukocyte sub-types when Western blotting was employed. Here we also experienced technical difficulties. The protein concentrations isolated from leukocytes were very low and as a result not many samples could be analyzed. However, the remaining samples were subjected to SDS-PAGE and volumetric analysis detected a significant increase in *O*-GlcNAc modified proteins in the diabetic samples (Figure 3.7). We propose that more sensitive changes were not detected due to the considerably lower sample sizes available.

Together these data clearly illustrate how HBP flux increases in response to excess glucose availability (pre-diabetic and diabetic individuals). Our findings therefore firmly support, with strong statistical power and three alternative techniques, the original premise of utilizing this as a novel diagnostic marker.

4.3 Differential leukocyte OGA expression between healthy, pre-diabetic and diabetic individuals

OGA catalyzes the removal of *O*-GlcNAc and, together with OGT, is responsible for regulating *O*-GlcNAc levels (reviewed in [193, 220]). OGA catalyzes the glycosidic bond between *O*-GlcNAc and target proteins, and previous work identified its supportive role for *O*-GlcNAcylation in the progression of type 2 diabetes (reviewed in [193, 259]). For example, mutations and splice variants of the OGA gene are associated with the onset of diabetes [203, 260]. Although the results for OGA expression include variances in sensitivity between the three characterizing categories, they all follow the same major trend, i.e. increased OGA expression in diabetic individuals.

For our total leukocyte data, diabetic individuals (HbA1c characterized) displayed significantly decreased OGA expression (Figure 3.10A). This was not surprising since we expected increased *O*-GlcNAcylation (refer Discussion section 4.3) to be accompanied by lower expression of the enzyme responsible for its detachment. However, OGA protein levels did not show any significant differences for subjects characterized according to FPG levels (ADA and WHO criteria) (Figure 3.8A and 3.9A). Interestingly, when re-categorized i.e. normal, pre-diabetic, diabetic (7-8 mmol/L) and severely diabetic (9-23 mmol/L), decreased OGA expression was

detected in diabetic individuals (WHO and ADA) and thus supported our observations with HbA1c (See Fig. B1 and B2 in Supplemental data, pg 173).

Conversely, the sub-population data (granulocytes and lymphocytes) revealed greater OGA expression in diabetic individuals compared to normal and pre-diabetic subjects (Figure 3.8B, 3.8C, 3.9C, 3.10B, 3.10C). Our supplemental data also (in part) support these findings as we found increased OGA expression in severely diabetic versus diabetic individuals (See Fig. B1 and B2 in Supplemental data, pg 173). These data were originally not included in the Results chapter of this thesis due to the atypical nature of making a distinction between diabetic (7-8 mmol/L) and severely diabetic individuals (9-23 mmol/L). However, due to the statistical significance of these differences and the seemingly contradictory results earlier obtained, we are of the opinion that these data are of great value. It also emphasizes the shortcomings of narrow boundary cut-off values (7-8 mmol/L) and its influence in potentially skewing the data.

Our findings are in agreement with previous work that found higher OGA expression in pre- and overt diabetic subjects [80]. Here the authors argued that augmented OGA levels in erythrocytes may represent an adaptive response [80]. It is well established that *O*-GlcNAc plays a major role in modulating proteins upon stress, regulating protein stability and the activity of enzymes to preserve cellular function [194, 261]. It is therefore rational to suppose that in response to chronic *O*-GlcNAcylation, cells would attempt to restore stability and the function of the cell by increasing OGA protein levels. Moreover, studies that pharmacologically or genetically increased *O*-GlcNAcylation observed undesired elevations in OGA expression [262, 263], therefore also supporting the concept of an adaptive response. Although our data remains inconclusive regarding the timepoint at which

such an adaptive response commences (requires further investigation), it supports the notion that increased leukocyte OGA expression in diabetic subjects is an attempt to diminish overall *O*-GlcNAcylation and thereby blunt its potential damaging effects.

OGA protein levels were also determined with immunofluorescence microscopy. Due to *O*-GlcNAcylation matching difficulties we did not detect any significant differences (Figure 3.8E and 3.9E and 3.10E). However, marked observational differences in OGA signal was reported when implementing immunofluorescence as a qualitative tool (Figure 3.8F and 3.9F and 3.10F). Here it must also be noted that there was a considerable discrepancy between "normal" and "pre-diabetic" classifications for HbA1c versus FPG characterized individuals. In fact, analysis of raw data ascertained that only 10.9% of the population was classified as "normal" according to HbA1c guidelines versus 31.1% and 41.5% by ADA and WHO criteria, respectively (further details to be discussed in section 4.8 of this thesis). Such conflicting characterizations resulted in too few control images available for quantification and hence the representation of only two groups in Figure 3.6E and 3.10E.

With Western blotting analysis increased OGA expression was detected in pre-diabetic and diabetic samples versus controls (Figure 3.11). Here diabetic individuals displayed significantly decreased OGA protein levels, suggesting perhaps the early initiation of an adaptive response with an ensued stabilization of this reaction in the diabetic group. However, such analysis was performed on a relatively small sample size (n=4-5) and these results should therefore be carefully interpreted.

Our data therefore suggest that, although uncertainty exists regarding when exactly the proposed adaptive response is initiated, OGA upregulation in diabetic subjects is statistically robust (Figure 3.8B,C,D; 3.9B,C,D; 3.10B,C,D). These observations encourage further investigation since increased OGA expression may also offer diagnostic utility.

4.4 OGT expression did not significantly change for any of the study recruits investigated

OGT is an evolutionarily conserved, soluble enzyme that is ubiquitously expressed in all multi-cellular eukaryotic organisms (reviewed in [191]). Its regulatory role is executed via an *O*-linkage and subsequent addition of a single *O*-GlcNAc moiety onto serine/threonine amino acid residues present in nuclear, cytoplasmic and mitochondrial proteins [227, 232, 264].

Since we found increased *O*-GlcNAcylation we expected a concomitant elevation in OGT levels, the enzyme responsible for the attachment of *O*-GlcNAc [219]. However, we found that OGT expression did not significantly change with pre- or overt diabetes (Figure 3.12). Park *et al.* (2010) also reported unchanged OGT protein levels with diabetes, thus supporting our data [80]. This may too represent an adapttive response. In order to protect the cell from the damaging consequences of increased HBP flux, leukocytes may be attempting to not over-modify proteins with the *O*-GlcNAc saccharide under conditions of chronic hyperglycemia (diabetes). However, Park *et al.* (2010) emphasized that the question whether OGT enzymatic activity in erythrocytes could be altered by increased glucose concentrations remains elusive [80]. This issue therefore also needs consideration in our leukocyte model.

Our results were further enlightened by findings illustrating that OGT is not only regulated by the concentration of its substrate (UDP-GlcNAc) [246], as this reaction is too gradual to initiate fleeting alterations in *O*-GlcNAcylation [247]. In fact, previous studies found that OGT is activated through alternative mechanisms that include phosphorylation by calcium calmodulin-dependent protein kinase IV (CaMKIV) [265] and its binding to inositolphosphate lipids [266]. Moreover, it is essential to recognize that although OGT is encoded by a single gene [191], this deceivingly simple enzyme is indeed rather complex. For example, it has three different isoforms, it can be post-translationally modified by several kinases, and OGT itself can also be *O*-GlcNAcylated [219, 224, 228, 267]. These complexities also need to be considered when investigating changes in OGT in response to hyperglycemia.

We experienced great difficulties to quantify OGT levels and we believe this is most likely due to the variability of the polyclonal antibody used (Abcam 50270, Cambridge, MA). Hence our sample size is not large enough to make any firm conclusions. We also explored use of an alternative OGT antibody (AL 28), obtained from Gerald Hart's Laboratory (Johns Hopkins University, Baltimore, USA) for Western blotting analysis. Although we detected a significant increase in OGT protein levels in diabetic subjects (Figure 3.13), variability and small sample size number (n=7-8) were still major concerns. Larger sample sizes and further optimization are therefore required to improve confidence in such data and also to ensure statistical accuracy.

4.5 Elevated *O*-GlcNAc/OGA ratios in pre-diabetic and diabetic individuals

An important objective of our exploratory study was to extensively investigate the diagnostic utility of *O*-GlcNAcylation. Due to the differential *O*-GlcNAcylation and OGA expression observed between healthy, pre-diabetic and diabetic subjects, we next determined the *O*-GlcNAc/OGA ratio. This is a unique ratio proposed by our laboratory and here the idea is to further broaden diagnostic utility.

Our results conclusively demonstrated that *O*-GlcNAc/OGA ratios increased with higher glucose and HbA1c levels in sub- and total leukocyte populations (ADA, WHO, HbA1c) (Figure 3.14-3.16). Despite variation in sensitivity, *O*-GlcNAc/OGA ratios could distinguish between normal and pre-diabetic, pre-diabetic and diabetic as well as normal and diabetic individuals. Thus increases in *O*-GlcNAc/OGA ratios may also provide an additional diagnostic avenue to be further explored.

4.6 Summary of main findings

Together our data validate the strong association between hyperglycemia (pre-diabetes and diabetes) and increased HBP flux, as well as its considerable potential for diagnostic value. The link between increased *O*-GlcNAcylation, OGA expression and *O*-GlcNAc/OGA ratios and the severity of glucose dysregulation is supported by solid statistical data and therefore represents a significant stride in improved diagnosis of diabetes.

In light of slight variations between some of our findings we are of the opinion that the following points should be considered:

1) Further investigations into *O*-GlcNAcylation of proteins found in granulocytes and lymphocytes are required to elucidate subtle differences in sensitivity. Moreover, determination of *O*-GlcNAcylation sites should provide valuable insight regarding the identity of specific proteins that are targeted in this process. However, these subtle differences may also be mitigated with an increase in population size.

2) It is important to note that the sample size of the pre-diabetic group was usually the lowest in this study. This is perhaps linked to one of the exact motivations for our study: the earlier detection of diabetes. Pre-diabetic individuals are difficult to identify with the current diagnostic tools [84] and are therefore the most difficult to recruit. We believe that the lower sample size considerably hampers statistical power and could explain subtle differences in pre-diabetic lymphocyte and granulocyte *O*-GlcNAcylation and OGA expression, and the uncertainty regarding when exactly the OGA adaptive response occurs.

3) Discrepancies between alternative defining criteria (ADA vs. WHO), and between diagnostic approaches (FPG vs. HbA1c) affected the characterization of our participants (to be further discussed in section 4.7.2). This may elucidate incongruities observed in our study, e.g. decreased OGA expression in HbA1c-characterized individuals [Figure 3.10]).

4) We are compelled to investigate the accuracy of our novel tool by characterizing study subjects based on the exact inaccuracies of tools we are aiming to advance/replace. We are of the opinion that such inaccuracies (especially with fasting plasma glucose) may also impact on the sensitivity of our assays and the

subtle discrepancies in statistical power between significant groups (normal vs. pre-diabetic or pre-diabetic vs. diabetic).

5) Although the alternative techniques employed (immunofluorescence microscopy and Western blotting) lacked strong statistical power, this is most likely due to their qualitative nature and/or the various technical difficulties incurred. It did nevertheless corroborate our flow cytometric data and thereby further strengthen our intriguing findings.

4.7 Further analysis into *O*-GlcNAc's diagnostic utility

4.7.1 Discrepancies between diagnostic tests and defining criteria

Crucial matters of concern in the field of diabetes are the various limitations of current diagnostic tools (reviewed in [49]). Not only are there discrepancies between preferred screening methods and alternate diagnostic thresholds, each diagnostic test have several idiosyncrasies that hamper the effective diagnosis of diabetes (extensively reviewed in the Introduction section 1.3.2 of this thesis). For example, results from FPG and OGTT tests have poor reproducibility and short-term variability in glycemic measurements [72]. OGTT is impractical and its utilization therefore limited in clinical practice and it is often used only to further evaluate individuals that already present with IFG or HbA1c levels between 5.7-6.4% [268]. HbA1c is affected by ethnicity, age, numerous erythrocyte anomalies and cannot be used for the diagnosis of gestational diabetes [269, 270]. Furthermore, differences between alternative diagnostic approaches in terms of diagnostic yields and predictive capacity further perpetuate the inefficient diagnosis of type 2 diabetes [271-273].

The abovementioned complexities formed a major part of the motivation for the inquisition into a novel marker for the more proficient detection of diabetes. Ironically, the exact complexities we were aiming to mitigate impeded the efficient characterization and investigation into *O*-GlcNAc's diagnostic utility. How could we accurately test *O*-GlcNAc's prognostic value without a recognized consensus regarding the definition of pre-diabetes, or which diagnostic approach was best at detecting it? Therefore the characterization of our study population into "three groups" became a relatively complex problem. Since OGTT tests were not feasible due to its cumbersome nature, this reduced our ability to comprehensively assess glucose metabolism in our participants. This double-edged sword intensifies the need for increased simplicity and efficacy in the field of diabetes diagnosis.

However, we enhanced the scope of our study by characterizing recruited individuals according to FPG (WHO and ADA criteria) and HbA1c. This proved valuable for the context of our study (and for diabetes diagnosis in general) as: ***1)*** *we could more extensively investigate our novel tool and determine its value in three current diagnostic settings;* ***2)*** *it underscored these discrepancies and the need for advancements in this field; and* ***3)*** *it provided an opportunity to assess discrepancies between diagnostic tests (and their consequences) in our recruited study population.*

The WHO and ADA both define diabetes on the basis of a fasting glucose concentration above 7 mmol/L [49]. An HbA1c level of >6.5% is also used for the diagnosis of diabetes [274]. Our results indicated that 13.6% of study recruits were either >7 mmol/L, <6.5% or <7 mmol/L, >6.5%. A recent study investigating HbA1c's utility in six different countries also detected major discordances [275]. Here the probability of individuals presenting with an FPG >7 mmol/L also meeting the HbA1c criteria of >6.5% varied between 17-78% [275]. These data exemplifies the repercussions of discordant criteria and the potential for misdiagnosis if either

the FPG, or HbA1c tests, are independently implemented. However, the dilemma is that for most countries it is not feasible to screen for diabetes with more than one test due to costs and availability (reviewed in [74]).

We therefore aimed to determine whether *O*-GlcNAcylation differentiated between these groups and analyzed *O*-GlcNAc levels of individuals representing: <7 mmol/L, <6.5%; >7 mmol/L, <6.5%; <7 mmol/L, >6.5%; and >7 mmol/L, > 6.5%. As expected, *O*-GlcNAc was significantly increased in the >7 mmol/L, >6.5% group (diabetic according to both sets of criteria) versus the <7 mmol/L, <6.5% group (normal by both sets of criteria). However, there was a significant difference in *O*-GlcNAcylation between the inconsistent groups (>7 mmol/L, <6.5%) and (<7 mmol/L, >6.5%) (Figure 3.17). These data thus further enhance *O*-GlcNAc's potential for diagnostic advantage and highlight its ability to reflect improved dynamic ranges of glycemia. This is a clearly favorable advance in light of the current status of diabetes diagnosis.

We next investigated discrepancies between FPG organization-specific criteria in our data. The WHO and ADA have a specific range of glucose concentrations (5.6-6.0 mmol/L) that allow for alternative classifications (refer to Results section 3.10.2). We found that 12.9% of our recruited individuals presented with glucose levels in this range. Thus they were characterized as normal according to WHO guidelines, but pre-diabetic according to the ADA criteria. After reviewing the literature, we could not find any studies investigating the ramifications of this differential characterization in a large-scale population. However, some studies showed that individuals presenting with FPG values of 5.6-6.0 mmol/L are associated with an increased risk for diabetes [54, 276].

We evaluated and compared *O*-GlcNAcylation levels in this group (5.6-6.0 mmol/L) versus the ADA normal (<5.6 mmol/L), ADA prediabetic (5-6-6.9 mmol/L), WHO

normal (<6.1 mmol/L) and WHO pre-diabetic (6.1-6.9 mmol/L) groups (Figure 3.18). Here we found significant increases in *O*-GlcNAc levels between ADA normal and pre-diabetic individuals, and WHO normal and pre-diabetic subjects. Moreover, there was a significant increase in *O*-GlcNAc modified proteins in the WHO pre-diabetics versus the ADA normal participants. Interestingly, the significant distinction between the (5.6-6.0 mmol/L) and the WHO pre-diabetic group could further strengthen *O*-GlcNAc's diagnostic probability. This together with the differentiation between <7 mmol/L, >6.5% and >7 mmol/L, <6.5% could contribute towards a more simplified diagnostic strategy.

4.7.2 Characterization of our study population

Due to the discrepancies discussed above, we also determined the effect of such inconsistencies on the distribution of normal, pre-diabetic and diabetic individuals in each category of characterization. The 31.1% of "normal" individuals classified by ADA guidelines increased to 41.5% when characterized by WHO criteria, owing to the (5.6-6.0 mmol/L) group previously discussed. Furthermore, a mere 10.9% of individuals were classified as "normal" when characterized according to HbA1c levels (Figure 3.19). This analysis helps to explain differences in the specificity of *O*-GlcNAcylation, OGA, and *O*-GlcNAc/OGA ratios between alternatively categorized groups. Nevertheless, the range of "normal" individuals between 10.9-41.5% is disconcerting.

Significant discrepancy also occurred between pre-diabetic groups, i.e. 22.1% of subjects were defined as "pre-diabetic" by ADA criteria compared to the 31.5% by HbA1c levels. The lowest proportion of pre-diabetes was presented in the WHO group (11.7%). The diabetic group remained the most constant as ADA and WHO criteria are identical in their classification of diabetes. Here 46.8% of subjects were

classed as “diabetic” compared to 57.6% according to HbA1c (Figure 3.19). These considerable differences between categories of characterization, particularly with regard to the normal and pre-diabetic groups, are a great concern. We believe these are the exact groups in which the most uniformity should exist as to most optimally improve prognostic strategies/outcomes and for the earlier detection/treatment of hyperglycemia-related complications.

After investigating the proportion of individuals characterized as normal, pre-diabetic or diabetic according to all three sets of characterization (“true”), we found that a significant proportion (38.75%) of our recruited participants remained uncharacterized (Figure 3.20). This effectively translates into more than a third of our study population being characterized into alternative groups when characterized by the separate sets of criteria.

We therefore evaluated if statistically stronger results would be obtained with the analysis of *O*-GlcNAcylation, OGA and *O*-GlcNAc/OGA ratios between only the “true” normal, pre-diabetic and diabetic individuals. Although significant increases in *O*-GlcNAcylation, OGA and *O*-GlcNA/OGA ratios were detected (Figure 3.21, 3.22, 3.23), the differences were not more sensitive than our current data. However, due to the elimination of essentially ~40% of our recruited individuals, the sample sizes were even further diminished and likely the explanation for the lack of robust statistical power. It would be interesting to compare the sensitivity of a “true” population of a more comparable size to the statistical strength of our collective data.

A further concern is the actual inaccuracies of the specific test results used for the characterization. The case study presented (refer Results section 3.13) demonstrated the inaccuracy of a once-off fasting glucose measurement. Two individuals’ FPG values differed by 0.9 and 2.1 mmol/L, respectively, from the

once-off reading compared to the five-year average reading. Thus these recruits were incorrectly identified as being pre-diabetic. Moreover, our calculations confirmed that if these two individuals were included in our pre-diabetic group, their lower *O*-GlcNAc levels would have skewed the mean *O*-GlcNAcylation calculated. This in turn would have resulted in a smaller difference between normal and pre-diabetic groups, thereby misrepresenting *O*-GlcNAc's diagnostic competence.

These data collectively epitomize the need for uniformity and standardization between tests and criteria, and also emphasize the difficulties we faced when attempting to best characterize our population. It is reasonable to speculate that these discrepancies could additionally have influenced our statistics and skewed the data to our detriment, desensitizing the differences observed and falsely weakening our results. However, we are of the opinion that this is a further testimony to the robust nature of *O*-GlcNAc, i.e. despite these inconsistencies we still obtained propitious results.

4.7.3 The value of insulin in diabetes diagnosis

The characteristic hallmark of type 2 diabetes, particularly pre-diabetes, is insulin resistance. As earlier reviewed, hyperglycemia is preceded by compensatory hyperinsulinemia [121]. During the early stages of insulin resistance the compensatory increases in insulin concentrations are capable of maintaining normal glucose levels. The concern therefore is that although insulin resistant, these individuals display FPG levels in the normal range and hence could be overlooked [116, 118].

Hyperinsulinemia is a mutual characteristic of Mexican Americans [277], Pacific Islanders [125] and African Americans [278], all ethnic groups with a high prevalence of diabetes (reviewed in [123]). Moreover, a study determined (follow-up period of 24 years) that basal insulin was the most competent predictor for the development of type 2 diabetes [116]. The most effective preventative strategy for diabetes should surely rely on the earliest intervention, and the point at which insulin levels rise prior to increased glucose concentrations seems most opportune. Therefore, *O*-GlcNAc's diagnostic worth may be criticized as its value has been shown to be reliant on increasing glucose levels. If we were to truly scrutinize the utility of *O*-GlcNAc we would have to ask: would a marker detecting increasing insulin levels, as opposed to glucose levels, instead be a more significant advance?

The immediate answer to this question is with an alternative one: why are insulin-based tests not currently employed as primary diagnostic strategies? Neither the IDF, WHO or ADA endorses any measures of insulin-resistance as official diagnostic or screening avenues [1, 4, 49]. The truth is that insulin resistance is elusive and thus diagnosis based on insulin resistance is clinically challenging [279]. Insulin testing is also expensive and the hyperinsulinemic-euglycemic clamp is impractical and not clinically accessible. Moreover, indices such as the homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) require measurement of both serum insulin and glucose levels, and depend upon variably complex calculations [279]. Thus serum insulin levels are poor indicators of insulin resistance and confer no clinical benefit [280], and this explains why general medical practice often excludes measures of insulin resistance.

Nevertheless, we could then ask: should mastering the shortcomings of such insulin tests be a priority over enhancing glucose-based tests? Is the duration of the normoglycemic-hyperinsulinemic state extensive enough to warrant concern?

We believe not. The state at which normal glucose concentrations are maintained by compensatory insulin secretion is short-lived as the pancreatic beta cells' ability to counteract the ever-increasing glucose and lipid levels is feeble [121]. Even in the early stages of hyperinsulinemia, mild increases in blood glucose levels do occur. However, due to concentrations remaining within the normal classification these subtle increases go unnoticed [115]. It is also proposed (hyperglycemia-pancreatic exhaustion hypothesis) that the pathology of diabetes includes a prolonged period of hyperglycemia and hyperinsulinemia (reviewed in [123]). This therefore helps to dispel concerns regarding increases in glucose being dependent on complete beta cell failure and therefore once established complications have already occurred. Thus although elevations in blood glucose occur after an initial rise in insulin levels, such increases occur early enough to waiver concerns regarding *O*-GlcNAc's dependence on the assessment of glucose metabolism. The pressing concern is therefore not the late-stage of glucose elevations, but the inability of the current tools to detect these increases prior to the onset of afflictions [84]. Therefore, it would be a significant step forward if *O*-GlcNAc could improve on this aspect.

Even if the limitations of detecting hyperinsulinemia were overcome, the accurate interpretation of insulin values would be particularly challenging. For example, the progression of pre-diabetes to diabetes would be envisioned by decreased insulin levels. However, the predicament is that although insulin levels are decreasing due to beta cell failure, this decline is progressive and the insulin concentrations remain high (relative to normal) [125, 281]. In fact, after gaining access to insulin

values for a subset of our individuals, we characterized our population according to glucose/insulin groups (refer Results section 3.14). When comparing the pre-diabetic and high glucose (diabetic) groups, the highest percentage of individuals were classified into the high insulin groups (PGHI, HGHI in Figure 3.24). We also found that for the HGNI group no diabetic subjects had insulin values <2.1 μU/mL. This illustrates that even during the later stages of glucose dysregulation insulin levels are simply relatively lower but remain "high" according to the reference ranges. This further depicts difficulties involved in interpreting relative changes in insulin values. In light of such problems and impracticalities of insulin measurements and increases in glucose occurring early enough [279, 280], we believe improving glucose-based assessments (e.g. *O*-GlcNAcylation) remains a promising avenue and provides a platform for significant advances to be made in diabetes diagnosis.

The perfect scenario for diabetes diagnosis would therefore ideally be an indirect, sensitive marker for elevations in both glucose and insulin levels. Could *O*-GlcNAc possibly make strides towards achieving this? Several *in vitro* and *in vivo* studies (reviewed in Introduction section 1.8) have validated *O*-GlcNAcylation as a roleplayer in regulating insulin signaling (reviewed in [193, 226]). The proposed model is that increased UDP-GlcNAc (due to enhanced HBP flux) upregulating *O*-GlcNAcylation and causes insulin resistance [184, 199, 202]. Therefore, it would be reasonable to hypothesize a link between *O*-GlcNAc metabolism and insulin levels.

We next determined if any changes in *O*-GlcNAcylation levels and/or *O*-GlcNAc/OGA ratios took place between the glucose/insulin groups. To our delight, there were significant differences in *O*-GlcNAcylation as well as in *O*-GlcNAc/OGA ratios between the (NGNI) and (NGHI) groups (refer Figure 3.24). This finding is particularly promising as the differentiation between glucose levels in the same

range (but with varying degrees of insulin) is the exact differentiation required for the earliest point of detection. Furthermore, previous work we performed in the earlier stages of our project demonstrated that *O*-GlcNAc could distinguish between varying levels of "normal" glucose metabolism. Of note, *O*-GlcNAcylation increased in the 4.8-5.5 mmol/L and 5.6-6.3 mmol/L groups compared to the 4.0-4.7 mmol/L, although not statistically significant (refer Fig. A1 and A2 in Supplemental data, pg 171-172). This demonstrates that *O*-GlcNAc may offer exceptional worth to detect subtle increases in glucose accompanied by early insulin resistance that usually remains unnoticed.

Although further studies are required to support these data, our findings indicate a possible link between *O*-GlcNAc and the detection of early elevations in glucose and insulin levels in the habitually concealed "normoglycemic hyperinsulinemic" condition, thereby contributing the ideal attributes of a novel prognostic tool.

4.7.4 The effect of insulin on HBP flux

To our knowledge there is very little literature elucidating the effects of insulin on the HBP. Thus we desired to additionally investigate insulin's influence on the HBP to potentially shed light on the differential *O*-GlcNAcylation observed between the various glucose/insulin groups (Figure 3.25).

We employed an H9c2 cardiomyoblast cell line model (refer Materials and Methods section 2.7.2), and determined the effect of different doses of insulin (0.1 μM, 1 μM, 10 μM) on *O*-GlcNAcylation with fluorescence microscopy and Western blotting. Here immunofluorescence analysis (qualitative) visually detected decreased *O*-GlcNAcylation in the high insulin groups, with both low glucose and high glucose treated cells (Figure 3.26 and figure 3.27). This demonstrates that high insulin

levels may play a role in decreasing *O*-GlcNAcylation. However, this result was not confirmed by Western blotting where we found no differences in *O*-GlcNAcylation between any of our experimental groups (Figure 3.28). However, the sample size used for both immunofluorescence microscopy and Western blotting were relatively small and insulin values were only acquired for a subset of our population.

The utilization of a cardiomyoblast cell-line may also not have been the most pertinent choice in the context of our study. The reason being that, unlike leukocytes, heart and muscle cells are insulin sensitive [208]. Several studies have demonstrated increased *O*-GlcNAcylation within heart muscle in diabetic models (reviewed in [190]) and although skeletal muscle is the major site of insulin resistance, it is less evident if identical mechanisms are present in cardiac muscle [90]. Moreover, hyperglycemia-induced activation of the HBP can result in myocardial cell death [168]. However, our hypothesis is based on the fact that insulin-dependent tissues (muscle, fat and liver) have a reduced capacity for glucose uptake (due to insulin resistance), resulting in hyperglycemia (pre-diabetes and diabetes), increased glucose uptake and subsequent HBP flux by insulin insensitive tissues [140]. Therefore, a cardiomyoblast cell line would likely display decreased glucose uptake in pre- and diabetic settings and therefore be an unsuitable model for our specific aim. We propose that these data be cautiously interpreted and that further studies be conducted in an insulin-independent cell line.

4.8 Matters in need of consideration

The following points of discussion are important for the context of this study and require attention:

1) We have provided strong evidence demonstrating *O*-GlcNAc's sensitive assessment of glucose metabolism in individuals with both subtle and overt glucose dysregulation. However, what exactly is the level of *O*-GlcNAcylation representing in terms of an individual's glucoregulatory status? For example, FPG represents a single facet of glucose metabolism (reviewed in [87]) and HbA1c reflects average blood glucose levels over the half-life period of erythrocytes (~2 months) [282]. Due to sensitive measures of *O*-GlcNAcylation only recently becoming more facile (reviewed in [192, 204]), certain aspects regarding *O*-GlcNAc's regulation are still poorly understood [228]. We can therefore only speculate regarding the features of glucose metabolism represented by *O*-GlcNAcylation.

O-GlcNAcylation is a more specific and regulated modification versus non-enzymatic glycation [87]. Moreover, the principal purpose of *O*-GlcNAcylation is the regulation of cellular activities in response to nutrients and cellular stress (e.g. hyperglycemia) [188, 223]. Therefore we propose that if exposed to sustained hyperglycemia (pre- and full-blown diabetes), increased leukocyte *O*-GlcNAcylation would reflect a chronic measurement of glucose metabolism as opposed to the glucometabolic status of a single time point. In support, Wang *et al.* (2009) hypothesized that alterations in erythrocyte *O*-GlcNAc-modified proteins would be capable of monitoring the history of glucose changes [87]. For this reason we also propose that *O*-GlcNAcylation would remain stable during acute bouts of hyperglycemia and could thus potentially also be utilized under non-fasting conditions (a major advantage of a diagnostic tool).

However, would *O*-GlcNAc's measure of glycemia be restricted to the various lifespans of WBCs? The lifespans of erythrocytes play a major role in HbA1c testing due to the slow, irreversible non-enzymatic reaction between glucose and hemoglobin [283]. However, an advantage of *O*-GlcNAcylation is that it is a reversible reaction [205] and therefore the various lifespans of leukocytes are not a concern. Unlike HbA1c, *O*-GlcNAcylation is regulated under the tight control of OGT and OGA [191, 226]. A valuable advantage is therefore that it can be dynamically attached and removed from proteins (cycling) dependent on nutrient availability [190, 225]. Therefore if an individual's glucose dysregulation improves (e.g exercise intervention), then *O*-GlcNAcylation would change and stabilize at a reduced level. This was supported by a study that observed decreased *O*-GlcNAcylation levels in swim-exercised mice [284]. In light of this it may be possible that *O*-GlcNAcylation would provide an accurate estimation of both the severity and period of glucose dysregulation [87]. Although it is imperative that this aspect be validated in future studies, we believe it reasonable to speculate that *O*-GlcNAcylation levels reflect stable, average measures of glycemia that can reflect fluctuations in broader and more dynamic ranges compared to current diagnostic tests.

2) It has recently been debated whether the detection of pre-diabetes is a worthwhile strategy [285]. This is an important matter for discussion as the impetus for this study (in part) was improved detection of pre-diabetes. What is pre-diabetes exactly? Pre-diabetes is defined as having IFG or IGT (refer Table 1.2) as this indicates a high risk for the development of diabetes [286]. Moreover, pre-diabetic individuals are at significant risk for micro- and macrovascular complications, independent of its progression to full-blown diabetes [286-288]. The determinants for the progression to diabetes are different in individuals with

isolated IFG and IGT [128] and post-prandial hyperglycemia is an independent risk factor for cardiovascular pathologies [57]. This highlights not only the necessity for pre-diabetes to be detected, but also the differentiation between the alternative glucose intolerance categories.

We believe that diabetes is not necessarily a definitive state, i.e. it should not only be considered as a condition that exists above a certain glucose threshold, but rather as a continuum of glucose dysregulation. The diagnosis of diabetes is to some extent arbitrary, a spectrum from health to diabetes, with the concurrent shift between low and high risk as glucose levels gradually increase [274]. Why define diabetes then? The inability to define diabetes efficiently has been the major issue since diabetes' description over 2000 years ago [50] and the fact that glucose is a continuous risk factor is most likely the reason for the on-going struggle and lack of consensus regarding its definition. The current scenario is that one trip across the Atlantic may cure or cause diabetes based on incongruities between definitions [50]. Defining diabetes has proven so difficult due to the continuum of severity lacking a finite point at which pathologies arise and medical intervention should commence [32]. Nevertheless, diagnostic cut-off values are required, and we encourage the continued and frequent revisions of criteria for both "pre-diabetic" and "diabetic" conditions in hope for the earlier and more standardized detection of glucose dysregulation.

3) We have extensively reviewed and highlighted the numerous and serious deficiencies and discordances with current diagnostic tests and defining-criteria. The poor compliance between HbA1c assays and glucose-based assessments is the result of such tools identifying distinct facets of glucose homeostasis. Therefore each test has its own technical merits as well as advantages in different aspects of

diabetes diagnosis (refer to Introduction section 1.3) [49, 289]. In light of this, choosing a single test for the most effective diagnosis is not plausible and therefore the current status of diabetes is considerably complex. A point of criticism might be that the proposal for the addition of another diagnostic tool will result in further intricacies, ambiguity and confusion.

It is likely that advancing a new marker from scratch could with time result in a more optimal endpoint than advancing well-established diagnostic tools. In support, the current screening assays have all been introduced and advanced over several decades and yet retain numerous shortcomings (reviewed in [32, 81]). By contrast, investigation into the utility of *O*-GlcNAcylation, even in its infancy, shows great promise for improved sensitivity and the differentiation between pre-diabetes and diabetes.

The ultimate goal would, however, be the implementation of a single diagnostic test. We believe that *O*-GlcNAcylation could be that test. Although further studies are required to support our results, our preliminary data demonstrated that a) the extent of *O*-GlcNAcylation is highly sensitive towards increased glucose concentrations, 2) *O*-GlcNAc metabolism is likely associated with both glucose and insulin levels, 3) *O*-GlcNAc could distinguish discordances between both WHO and ADA FPG criteria, as well as between FPG and HbA1c tests. Furthermore, the robust regulation by OGT and OGA and the stimuli-responsive nature of *O*-GlcNAc may allow for the dynamic and stable glucometabolic assessment. Moreover, the strong association between *O*-GlcNAcylation and diabetes-associated complications indicates the potential for powerful predictive capacity [192, 193, 290]. The integration of these aspects could therefore contribute to a more simplified diagnostic approach.

4) We would lastly like to raise awareness regarding the importance of translational studies such as this one. The phrase "bench to bedside" is a term often used and heard, but is it truly implemented? The understanding of disease mechanisms and the innovation of biomedical advances in the laboratory is only the first step, though equally important is the actual movement of such innovations into real-world medical practice [291]. Huge amounts of time and funding are spent on broadening the understanding of intricate biological mechanisms and pathways underlying diseases, but far less focus is applied to the utilization of this knowledge toward advances that will actually improve the quality of life of those millions burdened by illness [292]. Why is this the case? It could be due to translational studies receiving less funding than basic science projects [293] as well as the fact that careers in science are competitive and associated with prestige and status [294]. Nevertheless, the limited attention devoted towards the transformation of basic science into clinical applications is a matter of concern, and the need for patient-orientated translational research is imperative for the reduction in health disparities and the improvement of global human health [295]. However, recent initiations by organizations such as the NIH to promote translational science are encouraging [296].

In our study we could utilize the changes in global *O*-GlcNAcylation without having to interpret the effects of these changes. Although the extensive investigation into these changes are vital, several studies are already determining this and we could therefore specifically focus on simply using such changes for the primary aim of clinical advantage. We are of the opinion that this strategy significantly increased the 'translation potential' and is particularly relevant due to the extreme severity of diabetes, thereby supporting the urgent need for a global response to this epidemic.

Although the importance of translational research has been increasingly recognized, in our experience the relationship between medical practitioners and scientists is considerably poor. The success of such studies rely on the integration between clinicians and researchers and therefore the severe lack of interest by health-care professionals contributed great difficulties toward the effective execution of this study. However, the initial/exploratory phase of our translational-motivated study epitomizes the tremendous potential of basic scientific knowledge and we encourage more studies to employ similar tactics.

4.9 Evaluation of techniques employed

The successful investigation into *O*-GlcNAc's diagnostic potential was accomplished with the implementation of four different techniques i.e. flow cytometry, immunofluorescence microscopy, Western blotting and cell culture. We have shown that the application and optimization of several techniques provided a vital platform to: a) corroborate our findings and ascertain its validity, thereby improving the statistical potency of our results; and b) enhance knowledge, develop practical skills, and improve overall understanding of fundamental principles underlying contributions to clinical sciences.

Determination of *O*-GlcNAcylation levels has traditionally been executed via Western blotting [252]. More recently, immunofluorescence microscopy also effectively identified *O*-GlcNAc modified proteins [240]. However, both Western blotting and immunofluorescence microscopy are technically demanding, time-consuming and provide only qualitative information [239]. Thus the implementation of sensitive, quantitative and simple tools for *O*-GlcNAc detection are lacking (reviewed in [190]) and flow cytometry is a good option to fill this void

[239]. Here we argue that flow cytometry is a rapid, and more quantitatively resourceful approach as it can easily analyze thousands of single particles/cells. Moreover, it is able to distinguish/separate cells into populations (as seen with our leukocyte data) based on up to 20 alternative variables [245]. Since the scope of such technology may have significant impact on clinical research, we employed it as our principal technique. Furthermore, since the development of novel diagnostic tools should ensure that analyses are clinically practical, i.e. accurate, quantitative and time-efficient, it is our opinion that a flow cytometric-based method provides diagnostic utility.

4.10 Shortcomings

As in all research, our study had limitations:

- The lack of 2-hour glucose concentrations resulted in the inability to distinguish between IFG and IGT.

- Insulin levels were only acquired for a subset of study recruits.

- Variability between flow cytometric data when samples were analyzed on different days or after the flow cytometer was serviced. This resulted in the loss of data and accounts for sample sizes not being identical throughout the Results section.

- Inadequate information regarding subjects' lifestyle choices and family history of diabetes, etc.

- Information on glucose-lowering medications was unavailable and the possibility of participants being on medication was not taken into consideration.

- Did not distinguish between different ethnic groups. This could prove important as alternative ethnic-specific reference ranges could result in increased sensitivity and specificity.

- Important factors such as gender and HIV status was not taken into consideration.

4.11 Future research

Future plans to enhance this study include the following:

- Most importantly, further investigations need to include a considerably greater sample size. Although our preliminary studies show great promise, the only approach to robustly determine whether *O*-GlcNAcylation is sensitive and specific enough to have significant value as a diagnostic tool, is with a much larger clinical sample size.

- The time-frame and stability of *O*-GlcNAcylation needs to be elucidated. We propose a long-term follow-up study where the same individuals are assessed for *O*-GlcNAcylation at various time intervals and under fasting as well as non-fasting conditions.

- Insulin, FPG, HbA1c and OGTT glucose levels need to be assessed for all individuals as to better determine the association of *O*-GlcNAc with insulin and the various categories of impaired glucose metabolism.

- To statistically establish *O*-GlcNAc reference ranges and to calculate its sensitivity and specificity. Also, to compare such *O*-GlcNAc criteria versus

HbA1c, fasting and 2-h post-load plasma glucose criteria in diagnosing diabetes in a large population of volunteers.

- To perform genetic analysis of OGA, OGT and GFAT expression in normal, pre-diabetic and diabetic individuals. Moreover, to analyze genes involved in the PPP, polyol and PKC pathways (also upregulated by hyperglycemia) to determine if these genes are differentially expressed with changes in glucose levels (this is currently being pursued by our research group).

- Determine site-specific *O*-GlcNAcylation in leukocytes and its various sub-populations using mass spectrometry.

- Determine the effect of immune-system disorders such as leukemia and AIDS on leukocyte *O*-GlcNAcylation.

Conclusion

Our initial investigation into the diagnostic utility of *O*-GlcNAcylation shows considerable potential. The early and significant increases in leukocyte *O*-GlcNAcylation (particularly leukocyte sub-populations) demonstrate the sensitive assessment of glucose metabolism in individuals with both subtle and overt glucose dysregulation. Elevations in OGA expression and *O*-GlcNAc/OGA ratios in pre-diabetic and diabetic individuals may also offer diagnostic worth. *O*-GlcNAc's detection potential was analyzed in total and sub-groups of leukocytes, thereby broadening the scope for several site-specific diagnostic opportunities.

This study has provided further clarity regarding the current status of diabetes diagnosis and also analyzed various discrepancies between current diagnostic tests and organization-specific cut-off values. Such analysis highlighted not only the critical need for improvements in the field, but more importantly provided a possible solution. Here our *O*-GlcNAc data show favorable results despite such discrepancies. *O*-GlcNAc's prognostic advantage was further enhanced by findings exhibiting sensitive changes in *O*-GlcNAcylation in response to subtle glucose elevations. We also identified a possible association with insulin levels.

O-GlcNAc has several attributes that favor its diagnostic abilities. It is therefore reasonable to speculate that this reversible, stimuli-responsive and tightly regulated modification could represent stable, average measures of glycemia reflecting fluctuations in broad and dynamic ranges. The strong association

between *O*-GlcNAcylation and various diabetes-associated pathologies indicate predictive capabilities, further strengthening its potential for prognostic power.

O-GlcNAc is therefore a credible candidate in the search for a novel marker that exhibits increased simplicity and the earlier and more efficient detection of type 2 diabetes. It must be re-iterated that this is a preliminary and exploratory study. We are not proposing that *O*-GlcNAcylation now be rolled out as a diagnostic tool as the field of *O*-GlcNAcylation is still in its infancy. However, our novel findings provide a strong impetus for further investigation with a much larger sample size and holds potential for eventual diagnostic utility. In conclusion, our study shows great promise for the contribution of significant advances in diabetes diagnosis and the eventual mitigation of this epidemic's devastating burden.

"…remember that what you now have was once among the things you only hoped for." – Epicurus (341 BC - 270 BC)

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Supplemental Data

Increased *O*-GlcNAcylation with increasing degrees of "normal" glucose metabolism:

O-GlcNAcylation was assessed in individuals with varying degrees of "normal" glucose levels. Although differences were not significant, *O*-GlcNAcylation increased moderately from (4.0-4.7) to (4.8-5.5), (4.8-5.5) to (5.6-6.3) and from (4.0-4.7) to (5.6-6.3) (Fig. A1). Moreover, an identical pattern was observed in both the granulocytes and lymphocytes where modest increases in *O*-GlcNAc-modified proteins were observed (Fig. A2). It should be noted that the standard error was large due to relatively small sample sizes employed.

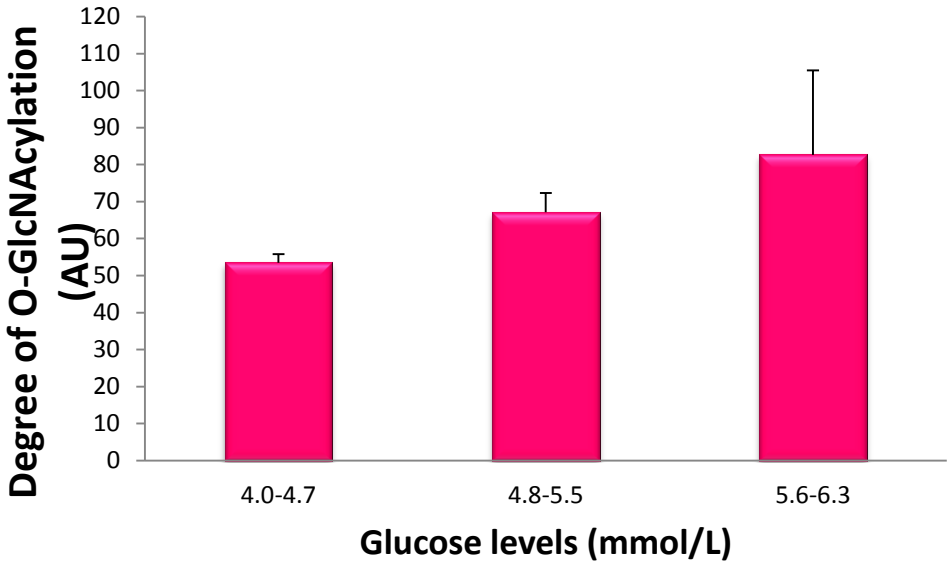

Fig. A1 *O*-GlcNAcylation is moderately elevated in the total white blood cell population of individuals with varying degrees of "normal" fasting plasma glucose levels. AU: Arbitrary units. Values are displayed as mean ± SEM.

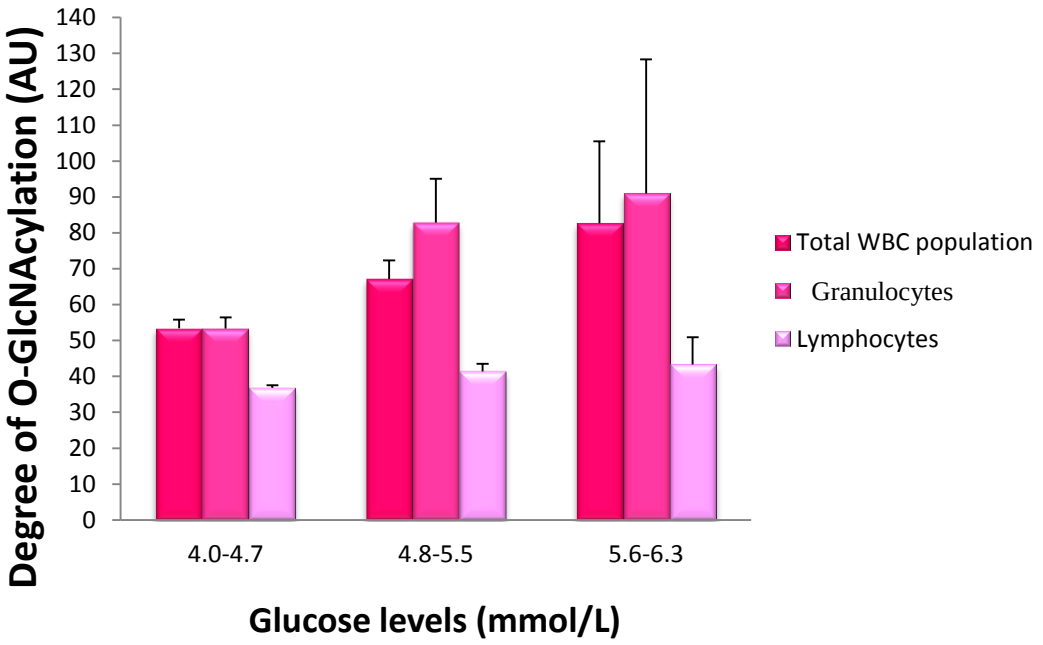

Fig. A2 *O*-GlcNAcylation is moderately elevated in granulocytes and lymphocytes of individuals with varying degrees of "normal" fasting plasma glucose levels. AU: Arbitrary units. Values are displayed as mean ± SEM.

Increased OGA expression in severely diabetic compared to diabetic individuals:

The raw OGA data (WHO and ADA) made a clear distinction between diabetic and severely diabetic individuals. When employing ADA criteria we determined that OGA expression decreased (not statistically significant) in the total leukocyte population in pre-diabetic individuals, and significantly decreased in diabetic subjects (Fig. B1). However, for the latter it was only apparent for individuals presenting with glucose levels between 7 and 8 mmol/L. Moreover, OGA protein expression differed significantly between diabetic (7-8 mmol/L) and severely diabetic individuals (9 - 23mmol/L), thus distinguishing between the two groups (Fig. B1). Here OGA protein levels decreased by 22.6 ± 4.7% in diabetic subjects, while significantly

elevated (26.9 ± 4.7%) in severely diabetic individuals versus diabetic subjects (Fig. B1). We observed an identical pattern when employing WHO criteria (Fig. B2).

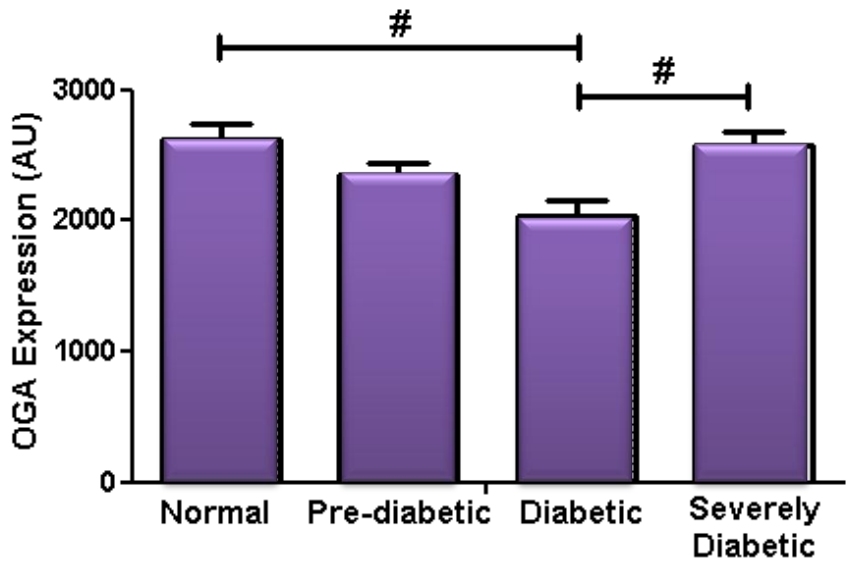

Fig. B1 OGA expression in total leukocyte population of ADA defined pre-diabetic, diabetic and severely diabetic subjects versus normal individuals (n=62, #p<0.05). AU: Arbitrary units. Values are displayed as mean ± SEM.

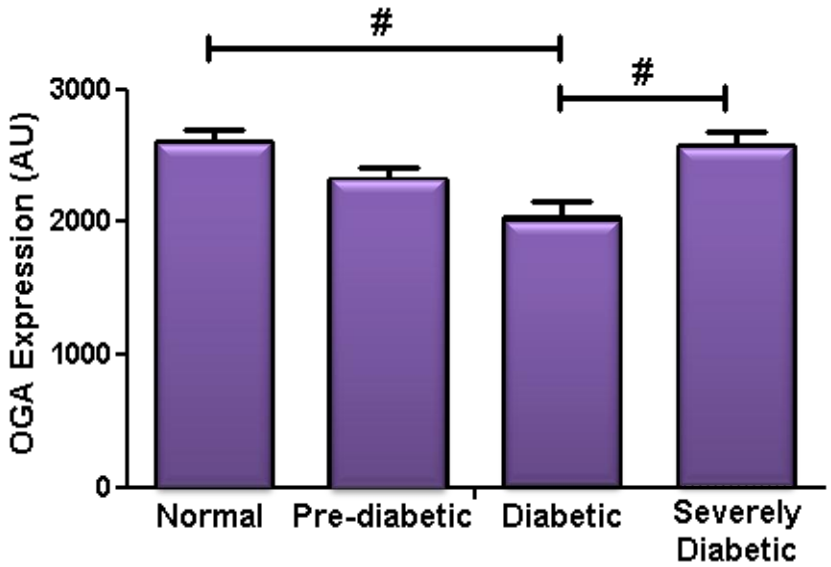

Fig. B2 OGA expression in total leukocyte population of WHO defined pre-diabetic, diabetic and severely diabetic subjects versus normal individuals (n=61, #p<0.05). AU: Arbitrary units. Values are displayed as mean ± SEM.

Appendix

Lysate preparation:

- Cell lysates, if frozen, should be defrosted slowly, on ice and kept on ice where possible for the remainder of the protocol.
- More PMSF can be added before cell lysates are sonicated using a Misonix S-4000 minisonicator (Misonix Inc, Farmingdale, NY).
- Sonicate lysates at an amplitude of 10, 3x5 seconds per sample, interspersed with 5 second intervals (5 seconds sonication, followed by 5 seconds rest, on ice if possible).

Bradford protein quantification:

Bradford reagent (5x concentrated):

- Dilute 500 mg of Coomassie Brilliant blue G in 250 ml 95% ethanol.
- Add 500 ml of phosphoric acid before mixing thoroughly.
- Make up to one liter with distilled H_2O (dH_2O).
- Filter and store at 4°C.

Bradford working solution:

- Dilute stock in a 1:5 ratio with dH_2O.
- Filter using 2 filter papers (at the same time).
- Solution should be a light brown color.

Bradford method:

- Thaw 1 mg/ml BSA stock solution.
- Thaw protein samples if in -80°C freezer. Keep on ice at all times.
- Make up a working solution of 100 µl BSA:400 µl dH_2O. Vortex mixture.
- Mark 7 microfuge tubes for the standards as well as tubes for the samples to be tested.
- Now add BSA and water to marker microfuge tubes as follows:

Blank:	0 µl BSA 100 µl dH_2O
2 µl protein:	10 µl BSA 90 µl dH_2O
4 µl protein:	20 µl BSA 80 µl dH_2O
8 µl protein:	40 µl BSA 60 µl dH_2O

12 µl protein:	60 µl BSA 40 µl dH_2O
16 µl protein:	80 µl BSA 20 µl dH_2O
20 µl protein:	100 µl BSA 0 µl dH_2O
Each sample:	0 µl BSA 95 µl H_20 5 µl of sample protein

- Briefly vortex all the tubes.
- Now add 900 µl of Bradford reagent to each microfuge tube. Vortex again.
- Let the solutions stand for ~5 minutes (switch on the spectrophotometer in the meantime).
- Read absorbencies, twice each, at 595 nm.
- If sample values fall outside the range of the highest standard then dilute with RIPA buffer.
- Make use of Excel to make a linear plot of absorbencies and then calculate the amount of each sample to be added to aliquots.

Sample preparation:

- Begin by setting beaker of water to boil.
- Remember to keep protein samples on ice at this point.
- Make up a stock solution containing 850 µl of sample buffer and 150 µl of mercaptoethanol.
- Vortex the solution.
- Calculate the number of sample sets needed, each containing one representative of each protein sample.
- Add sample buffer to each aliquot (do so under the fume hood to avoid exposure to harmful fumes).
- Add a volume of sample buffer equal to 1/3 of the final volume.
- Now add the amount of sample calculated previously to each respective microfuge tube.
- Punch small pin size hole in each tube then place in boiling water to stand for a period of 5 minutes.
- Spin tubes for a moment (~5 seconds) using the tabletop centrifuge.
- Samples can now be stored at -80°C.

Use of samples:

In the case that samples have been stored in the -80°C freezer:

- Start by bringing a beaker of water to the boil.
- Remove samples from the freezer.
- Make sure that small pin size holes have been punched in the top of each tube.
- Place in boiling water for a period of 5 minutes.
- Spin down momentarily (20 seconds) on the tabletop centrifuge (take care not to over centrifuge, especially if samples have been obtained from tissue.
- Samples can now be used for Western blotting.

Modified RIPA Buffer:

A 100 ml modified RIPA buffer contains:

- 50 mM Tris-HCl (790 mg of Tris in 75 ml distilled water and 900 mg of NaCl and pH made 7.4 using HCl).
- 10 ml of 10% NP-40 [final concentration 1%]
- 2.5 ml of 10% sodium deoxycholate [final concentration 0.25%]
- 1 ml of 100 mM EDTA pH 7.4 [final concentration 1 mM]
- Protease inhibitors (which include:)
    - 500 µl of 200 mM phenylmethylsulfonyl fluoride (PMSF) [final concentration 1 mM]
    - 100 µL of leupeptin (1 mg/ml water) [final concentration 1 µg/ml]
    - 80 µL of SBT1 (5 mg/ml water) [final concentration 4 µg/ml]
    - 100 µL of benzamidine (1 M) [final concentration 1 mM]
- Protein phosphatase inhibitors
    - 500 µL of 200 mM activated sodium orthovanadate (Na2VO3) [final concentration 1 mM]
    - 500 µL of 200 mM NaF [final concentration 1mM]
- 1 ml Triton X-100

This buffer is then made up to a final volume of 100 ml with distilled water and stored at -20°C.

Cell harvesting:

- Cells are washed with ice cold PBS.
- Add PMSF to RIPA buffer to a final concentration of 1 mM
- Add ~350-500 µl RIPA to each T25 flask and leave for 10 minutes.
- Use a cell scraper to lift the adhering cells from the surface of the flask.
- Transfer the cell lysates into microfuge tubes and either proceed with the protocol. Alternatively, store the lysates at -20°C for no longer than 2 weeks, or at -80°C for longer-term storage.