Investigating Differential Non-oxidative Glucose-utilizing Pathway Gene Expression as a Novel Diagnostic Tool for Type 2 Diabetes

Megan Coomer

Dissertation presented for the Degree of

Masters

In Physiological Sciences at Stellenbosch University

Supervisor: **Professor MF Essop**

December 2013

Cardio-Metabolic Research Group

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless of the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:……..

Date: ……

ABSTRACT

Context: Despite the availability and accessibility of current diagnostic tools, diabetes remains largely under-diagnosed. Biological limitations, discordant assays and conflicting diagnostic thresholds together impede the accurate and successful diagnosis of diabetes, providing impetus into research for a novel diagnostic tool.

Aim: Since flux through the five minor glycolytic pathways is increased during hyperglycemia, we hypothesized that the genes encoding the regulatory enzymes of such pathways may be differentially expressed between control, pre-diabetic and diabetic individuals setting the scene for an exploratory diagnostic avenue employing genetic biomarkers.

Experimental procedures: Participants (n=60; n=20 Mixed Ancestry, n=40 Caucasian) were recruited from Stellenbosch and Paarl (Western Cape, South Africa) and classified as control, pre-diabetic or diabetic. RNA was purified from leukocytes isolated from blood samples and *OGT*, *OGA*, *GFPT1, GFPT2, TKT, TKTL1* and *AKR1B1* expression determined by quantitative real-time PCR.

Results: Expression of *OGA, OGT, GFPT2* and *TKTL1* decreased in pre-diabetic and diabetic individuals; while *GFPT1, TKT* and *AKR1B1* expression levels remained largely unaffected between the study groups. *GFPT2* exhibited ethnic-dependent regulation.

Conclusion: Differential expression of genes regulating non-oxidative glucose-utilizing pathways may offer diagnostic utility in the future and warrant further investigation.

UITREKSEL

Konteks: Teen spyte van die beskikbaarheid en toeganklikheid van huidige diagnostiese instrumente, word diabetes nogsteeds min gediagnoseer. Altesaam belemmer biologiese beperkings, teenstrydige toetse en botsende diagnostiese grense die akkurate en suksesvolle diagnose van diabetes, wat bydra tot die druifkrag in navorsing vir ‘n nuwe diagnostiese instrument.

Doelstelling: Aangesien die vloei deur die 5 mineur glikolitiese pad weë toeneem gedurende hiperglukemie, veronderstel ons dat die gene wat regulatoriese ensieme kodeer in hierdie pad weë mag dalk differensieel uitgedruk word tussen kontrol, voor- en, diabetiese individue wat die toneel skep vir ‘n ondersoekende diagnotiese laan wat gebruik maak van genetiese merkers.

Materiale en metodes: Deelnemers (n=60; n=20 Gemengde afkoms; n=40 Blankes) was uit Stellenbos en die Paarl gewerf (Wes-Kaap, Suid-Afrika) en geklassifiseer as ‘n kontrol, voor- of diabeties. RNS was uit witbloodselle gesuiwer wat eers uit bloed monsters geisoleer was en *OGT, OGA, GFPT1, GFPT2, TKT, TKTL1* en *AKR1B1* uitdrukking was bepaal deur kwantitatiewe RT-PKR.

Resultate: Uitdrukking van *OGA, OGT, GFPT2* en *TKTL1* het afgeneem in voor- en diabetiese individue; terwyl *GFPT1, TKT* en *AKR1B1* utidrukkings vlakke meestal onaangeraak was tussen die studie groepe. *GFPT2* het etiese-afhanglike regulasie vertoon.

Gevolgtrekkings: Differensiele uitdrukking van gene wat glukose gebruik in nie-oksidatiewe pad weë reguleer bied diagnotiese gebruikbaarheid in die toekoms en bevel verdere ondersoek.

ACKNOWLEDGEMENTS

I show gratitude to my supervisor, Professor Essop, for his unwavering dedication to our research group, guided support over the past two years, trust, insight, and sunny disposition. Prof you are a true example of a well-rounded individual and I thank you for reminding me to fulfil aspects of my life that reach beyond the lab. Thank you for giving me the opportunity to be part of such a dynamic research group while simultaneously allowing me freedom and independence in my research. I am grateful for all this and more.

To my parents, thank you for allowing me the opportunity to stay at university for 7 years. Mom, I am so aware of everything you went without so that I could be afforded this chance and I am eternily indebted to you for that.

To my friends in the lab: Clare, Danielle, and Grete. Thank you for making the past years so memorable. Clare – thank you for being "my person" and for truly understanding me. Danielle – your positivity, unrelenting work – ethic, encouragement, and help will never be forgotten. Grete – thank you for being the "man" in our friendship – I know I can always come to you to solve my computer problems, for a good laugh, and of course to open my water bottles.

Thank you to the Stewart family for giving me a home away from home over the past three years and a comfortable environment in which to write my thesis and so much more. I am truly grateful to be so included in your wonderful family.

CMRG – it was so stimulating and refreshing to be part of such a dynamic group. The best in the department by far!

Thank you to the NRF for financial support.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

%:	Percentage
°C:	Degrees Celsius
3':	3 prime
5':	5 prime
A:	adenosine
ACTB:	Beta actin
ADA:	American Diabetes Association
ADP:	Adenosine diphosphate
AGE:	Advanced glycation end products
AKR1B1:	Aldo-keto reductase family 1, member B1
AKR1B1:	Aldose reductase
AKT:	Protein kinase B
ATP:	Adenosine triphosphate
BC:	Before Christ
BMI:	Body mass index
bp:	Base-pair
C:	Cytosine
cDNA	Complementary deoxyribonucleic acid
CoQ:	Coenzyme Q
CVD:	Cardiovascular diseases
Cyt C:	Cytochrome C
DAG:	Diacylglycerol

dH_2O:	Distilled water
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
ETC:	Electron transport chain
EtOH:	Ethanol
F:	Forward primer
FAD:	Flavin adenine dinucleotide (oxidized)
FADH2:	Flavin adenine dinucleotide (reduced)
FFA:	Free fatty acid
FOXO:	Forkhead transcription factor box protein
FPG:	Fasting plasma glucose
G:	Guanosine
G-6-Pase:	Glucose-6-phosphatase
G6PD:	Glucose-6-phosphate dehydrogenase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
gDNA:	Genomic DNA
GFAT/ GFPT:	Glutamine:fructose-6-phosphate aminotransferase
GlcN-6-P:	Glucosamine-6-phosphate
GLUT4:	Glucose transporter 4
GSK3:	Glycogen synthase kinase 3
GUS:	Beta-glucuronidase
GWAS:	Genome-wide association studies
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HbA1c:	Glycated hemoglobin

HBP:	Hexosamine biosynthetic pathway
HIV-AIDS:	Human immunodeficiency virus-acquired immunodeficiency syndrome
IDF:	International Diabetes Federation
IEC:	International expert committee
IFG:	Impaired fasting glucose
IGT:	Impaired glucose tolerance
IR:	Insulin receptor
IRS-1:	Insulin receptor substrate 1
Kb:	Kilobase
LRE:	Linear regression of efficiency
MGEA5:	Meningioma expressed antigen 5
MIQE:	Minimum information for publication of quantitative real-time PCR experiments
mmol/l:	Millimoles per litre
MODY:	Maturity-onset diabetes of the young
MONW:	Metabolically obese, normal weight
mRNA:	Messenger ribonucleic acid
NAD+:	Nicotinamide adenine dinucleotide (oxidized)
NADH:	Nicotinamide adenine dinucleotide (reduced)
NADP+:	Nicotinamide adenine dinucleotide phosphatase (oxidized)
NADPH:	Nicotinamide adenine dinucleotide phosphatase (reduced)
NEFA:	Non -esterified fatty acids
NFK-B:	Nuclear factor kappa-beta
ng/µl:	Nanogram per microliter

NO:	Nitric oxide
NOGPs:	Non-oxidative glucose-utilizing pathways
O_2^-:	Superoxide
OGA:	*O*-GlcNAcase
O-GlcNAc:	*O*-linked-N-acetylglucosamine
OGT*:*	*O*-linked β-*N*-acetylglucosaminyl transferase
OGTT:	Oral glucose tolerance test
OH:	Hydroxyl
OX:	Oxidized
PARP:	Poly(ADP-ribose) polymerase
PCR:	Polymerase chain reaction
PDE3B:	Phosphodiesterase 3 B
PDH:	Pyruvate dehydrogenase
PEPCK:	Phosphoenolpyruvate carboxykinase
PFK:	Phosphofructokinase
PI3K:	Phosphatidylinositol 3-kinase
PIP3:	Phosphatidylinositol 3,4,5-triphosphate
PKC:	Protein kinase C
PPP:	Pentose phosphate pathway
PTEN:	Phosphatase and tensin homolog deleted on chromosome 10
PTM:	Post - translational modification
PTP-1B:	Protein-tyrosine phosphatase 1B
q:	Long arm of chromosome
qPCR:	Quantitative real-time Polymerase Chain Reaction
R:	Reverse primer

RED:	Reduced
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RPL37A:	Ribosomal protein 37A
SEM:	Standard error of the mean
SES:	Socio-economic status
SHP2:	SH2-containing tyrosine-protein phosphatase
SOCS3:	suppressor of cytokine signaling 3
SOD:	Superoxide dismutase
SREBPs:	Sterol regulatory element-binding proteins
T:	Thymidine
T_A:	Annealing temperature
TB:	Tuberculosis
TBE:	Tris-borate/EDTA
TCA:	Tricarboxylic acid
TKT:	Transketolase
TKTL1:	Transketolase-like 1
TKTL2:	Transketolase-like 2
T_M:	Melting temperature
TPR:	Tetratricopeptide repeats
UCP:	Uncoupling proteins
UDP:	Uridine diphosphate
UDP-GlcNAc:	Uridine diphosphate *N*-acetyl glucosamine
UTP:	Uridine triphosphate
V:	Volts

WHO:	World Health Organization
β-cell:	Beta cell

LIST OF FIGURES

LIST OF TABLES

Chapter 1

LITERATURE REVIEW

1. LITERATURE REVIEW

The rapid increase in the incidence and prevalence of type 2 diabetes and its associated complications is fast becoming one of the most pressing health concerns of developed and developing countries (1). For example, diabetes is responsible for ~4.6 million deaths annually and this figure is predicted to increase by more than 50% over the next decade (2), (1). Additionally, diabetes healthcare expenditure totalled a considerable ~$465 billion in 2011, making it not only a global health affliction, but also a worldwide economic burden (2). Since the burden of diabetes is progressively shifting towards younger working class individuals, the associated economic repercussions are predicted to result in marked increases in expenditure (3). Moreover, non-communicable diseases such as cardiovascular diseases (CVD) and diabetes account for ~2/3 of all deaths worldwide (4). As a result the United Nations General Assembly proclaimed diabetes and other, non-communicable diseases a global epidemic in 2011 (5). Thus it is imperative that actions be taken in order to decrease the burden that diabetes places on society, our current healthcare structures, as well as its debilitating effect on the economy.

1.1 A GROWING GLOBAL EPIDEMIC

During 2000 the World Health Organization (WHO) revealed that there were ~171 million people suffering from diabetes globally, and predicted that this figure would increase to ~366 million by the year 2030 (6). However, these predictions have been well surpassed as there are presently ~346 million people suffering from diabetes and projections show that this figure will reach in excess of half a billion by 2030 if strategies are not put in place to deal with the growing prevalence of diabetes (7). This gross underestimation is of great concern and highlights the fact that diabetes has reached epidemic proportions and requires immediate

and urgent attention. Moreover, initially considered a condition associated with relative affluence, diabetes is now more prevalent in low- and middle-income countries with a predicted 69% increase in incidence from 2000 to 2030 compared to a projected 20% rise in developed countries (1). These compelling data led to diabetes earning the title of "global epidemic" as no country seems barred from its unrelenting upward trajectory (see Figure 1.1).

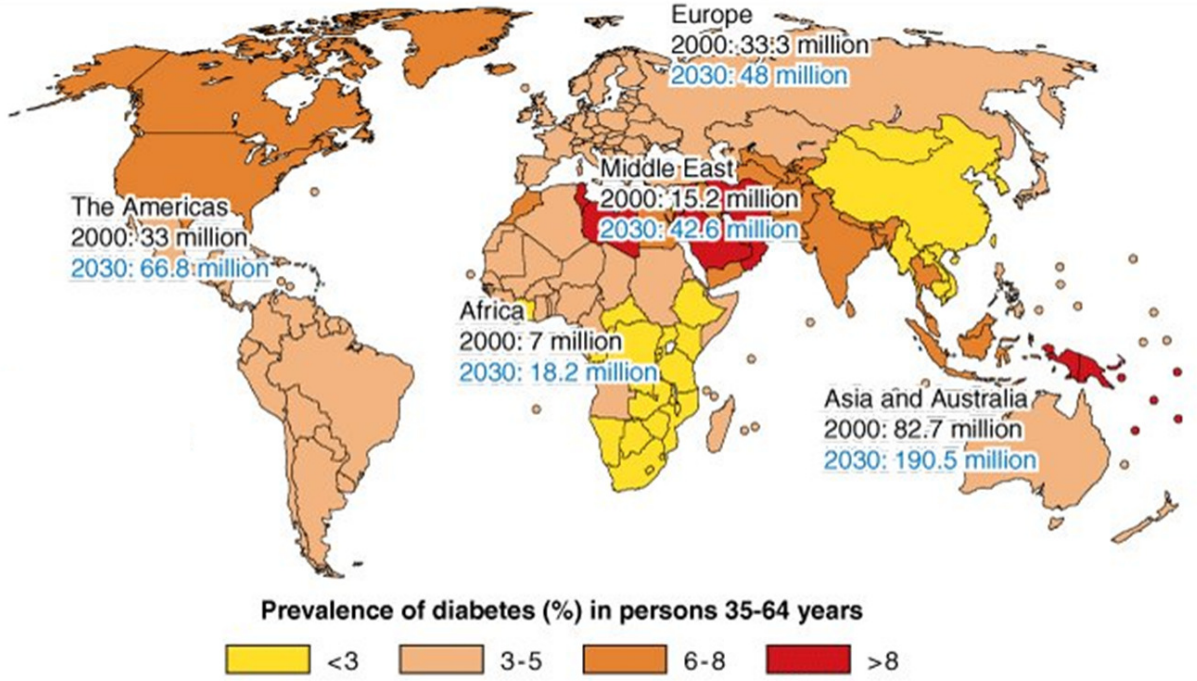

Figure 1. 1 Global diabetes prevalence in 2000 (black) and projections for 2030 (blue) shown by geographical area (6).

The African continent finds itself in a unique situation when viewed in this particular context. Already plagued with infectious diseases such as Human immunodeficiency virus-acquired immunodeficiency syndrome (HIV-AIDS) and tuberculosis (TB), Africans are facing a "dual-burden" of disease where non-communicable diseases such as diabetes and CVD are increasing in parallel (8). There are ~14.7 million reported diabetes cases in sub-Saharan Africa and this figure is projected to rise by a staggering 98% by 2030 (9). Since diabetes can exacerbate HIV-AIDS (10) as well as the development of active TB (11) the situation

becomes critical. Moreover, 80% of all diabetes-related deaths occur in low- and middle-income countries such as those typically found in Africa (4). Furthermore, the progression of diabetes on the African continent and other developing countries is heightened by poverty, socio-economic circumstances, and increased urbanization, culminating in a host of poor lifestyle choices together with the onset of obesity (4) (9).

Obesity remains a key driving force in the development of type 2 diabetes (12) and if current trends continue, an estimated 2 billion individuals will be classified as overweight by 2030 (13). However, in many Asian countries where the prevalence of obesity is relatively low there is a surprisingly high incidence of type 2 diabetes (14) (15). This suggests that other factors may also play a role in the onset and development of type 2 diabetes (refer to section 1.4.1 for further discussion).

1.2 DIABETES-ASSOCIATED PATHOLOGIES

Diabetes is associated with a plethora of micro- and macrovasculature complications manifesting in a variety of pathologies including; CVD (16), retinopathy (17), nephropathy and neuropathy (reviewed in (18)). This in turn causes an increase in morbidity and mortality rates among diabetic patients (19). Since CVD is the leading cause of death worldwide its close association with diabetes is of great concern (4). For example, 65% of all diabetes-related deaths arise from CVD. These statistics highlight the immense challenges encountered by diabetic patients, e.g. such individuals have on average a 50% higher chance of all round mortality when compared to non-diabetic persons (19).

Since the early diagnosis of diabetes attenuates the progression of the associated complications and consequently promotes a better patient outcome, it is imperative to promote efforts to increase the diagnosis of diabetes (20). This is especially pertinent to the diagnosis of pre-diabetes so that treatment regimens can be implemented before debilitating and costly complications are allowed time to develop. Such efforts should in turn help reduce some of the detrimental social and economic consequences associated with type 2 diabetes (reviewed in (19) (21)).

1.3 DIAGNOSIS

Despite the availability and accessibility of current diagnostic tools a substantial number (30 - 50%) of individuals remain undiagnosed (22). Furthermore, the prevalence of individuals with evidence of complications at diagnosis is worrisome (23) since this causes a tremendous number of unnecessary morbidity and mortality cases (2) (24). This supports the idea for increased efforts into earlier detection of diabetes (2) (24) since this should promote favorable patient outcomes and also be economically beneficial (19).

1.3.1 The Past

Diabetes was first recognized and described as early as ~600 BC (25). Initially the diagnosis thereof relied upon the attraction of ants towards a urine sample, and later the sweetened taste of urine (25). Since then considerable advances have been made, e.g. the introduction of biochemical assays such as the oral glucose tolerance test (OGTT), the fasting plasma glucose test (FPG) and most recently the measurement of glycosylated hemoglobin (HbA1c) in erythrocytes (26) (27).

Despite these clinical advances, the diagnosis of diabetes remains controversial as it is confounded by an on-going global debate regarding the preferred screening methods and organization-specific cut-off criteria/ diagnostic thresholds (28). For example, specific cut-off criteria have been repeatedly revised and amended by the American Diabetes Association (ADA) and the WHO; two leading expert organizations responsible for diabetes diagnosis (28) (refer to Table 1.1 for diabetes diagnosis amendments throughout the period 1985 - 2003).

Table 1.1. WHO *vs.* ADA diagnostic criteria: 1985 - 2003.

	OGTT (mmol/l)	FPG	OGTT (mmol/l)	FPG
	1985 WHO Criteria		**1999 WHO Criteria**	
Normal	<7.8	-	<7.8	<6.1
Pre-diabetes	7.8-11.0	-	7.8-11.0	6.1-6.9
Diabetes	≥11.1	≥7.8	≥11.1	≥7.0
	1997 ADA Criteria		**2003 ADA Criteria**	
Normal	Not endorsed as a diagnostic tool	<6.1	<7.8	<5.6
Pre-diabetes		6.1-6.9	7.8-11.0	5.6-6.9
Diabetes		≥7.0	≥11.1	≥7.0

ADA: American Diabetes Association; FPG: Fasting plasma glucose; OGTT: Oral glucose tolerance test; WHO: World Health Organization

1.3.1.1 Glucose based assays - OGTT (impaired glucose tolerance) and FPG (impaired fasting glucose)

Since hyperglycemia is the primary biochemical hallmark of diabetes, the diagnosis thereof has for decades relied upon the measurement of glucose levels using either the OGTT or the

FPG test (28). Each test requires an overnight fast with consequent blood glucose measurements. The OGTT requires the patient to ingest 75 grams of glucose orally whereafter blood glucose levels are measured over a 2 hour period. By contrast, the FPG test is a once-off blood glucose measurement (29).

The OGTT and FPG tests are also able to define an intermediate group of individuals whose glucose levels do not meet the criteria to be classified as diabetic, but are at a higher risk of developing diabetes than those with normal glucose levels (30) (31). This is referred to as impaired glucose tolerance (measured by the OGTT) or impaired fasting glucose (measured by the FPG test) (30). Such patients are referred to as having pre-diabetes and are at high risk of developing diabetes (30). While the WHO has traditionally favored the use of the OGTT the ADA has given preference to the FPG test, only endorsing the use of the OGTT to diagnose diabetes in 2003 (32). These tests were amended and revised throughout the period 1985 - 2010 in order to increase the specificity and sensitivity of diabetes diagnosis (28) (30) (see Table 1.2). Although both tests have consistently been used to diagnose diabetes for decades, there is less than 100% concordance between the 1999 WHO and 2003 ADA diagnostic criteria (33).

1.3.1.2 The controversial inception of HbA1c

In 2009 an International Expert Committee (IEC) convened by the ADA authorized HbA1c as an additional tool for the diagnosis of diabetes (27). That was acknowledged two years later by the WHO (34). However, HbA1c is not officially regarded as superior to blood glucose methods, but instead provides an alternative thereby adding further complexity to the already multi-faceted diagnosis of diabetes (27). The HbA1c test measures the level of glycated hemoglobin (glucose attached to various amino groups of hemoglobin) over the 120

day lifespan of an erythrocyte, reflecting a more stable, average measurement of glucose regulation (35). The IEC recommended a diagnostic cut off value of >6.5% to diagnose diabetes and this was later amended to include a pre-diabetic category (5.7 – 6.4%) (27).

1.3.2 The Present

1.3.2.1 Current ADA and WHO diagnostic criteria

Irrespective of the on-going global debate surrounding the diagnosis of diabetes, the 2010 ADA guidelines and 2011 WHO report together approve the use of the OGTT, FPG test and HbA1c assay to accurately diagnose diabetes (34). Notably, the pre-diabetes criteria differ between the two organizations with the WHO disregarding HbA1c entirely as a tool to identify this condition (36). Neither institution endorses the use of one test over another, and the decision regarding the most appropriate assay to use is left exclusively to the discretion of healthcare professionals (37).

Table 1.2. Current diagnostic criteria as specified by the ADA and WHO.

		ADA 2010	WHO 2011
OGTT (mmol/l)	Normal	<7.8	<7.8
	Pre-diabetes	7.8-10.9	7.8-10.9
	Diabetes	≥11	≥11
FPG (mmol/l)	Normal	<5.6	<6.1
	Pre-diabetes	5.6-6.9	6.1-6.9
	Diabetes	≥7	≥7
HbA1c (%)	Normal	<5.7	-
	Pre-diabetes	5.7-6.4	-
	Diabetes	≥6.5	≥6.5

ADA: American Diabetes Association; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; OGTT: Oral glucose tolerance test; WHO: World Health Organization

Despite the continual revision and amendment of diagnostic thresholds, incongruity between the ADA and the WHO persists, while discordance between the OGTT and FPG test remains. For example, in a large epidemiological study only 22% of newly diagnosed diabetics met the FPG criteria, 47% the OGTT criteria, and only 30% of individuals fulfilled both sets of diagnostic criteria (38). Despite the implementation of HbA1c reference ranges, many studies assessed the ability of the HbA1c assay to accurately diagnose diabetes (specificity and sensitivity) with various cut-off values yielding conflicting results (39). Moreover, in a study employing an HbA1c threshold value of >6.5% to diagnose diabetes only 11.2% of newly diagnosed individuals (glucose detection methods) were identified as being diabetic (38). So although an HbA1c threshold of >6.5% may demonstrate high specificity there is a trade-off with sensitivity, often missing individuals who are truly diabetic (40). Hence an HbA1c level of >6.5% used in isolation to diagnose diabetes should be interpreted with caution in order to prevent misdiagnosis. For this reason the WHO does not endorse the use of HbA1c to define pre-diabetes nor does it eliminate the possibility of diabetes diagnosis with an HbA1c level <6.5% (36).

Taken together it is clear that the diagnosis of diabetes is a complex process and cut-off criteria need to be constantly reviewed and revised to ensure the accurate and sensitive diagnosis of diabetes. Furthermore, no single test appears superior to another as each tool is subject to its respective advantages and limitations (refer to section *1.3.2.2*) and consequently alludes to the use of a combination of available tools. Regrettably, this is not always practical within the clinical setting.

1.3.2.2 Advantages and limitations of current diagnostic tools

Many factors can impede the reliability and reproducibility of diagnostic tests and it is thus important to consider these. Variability can stem from biological differences, the nature and sampling of the specimen, and the particular chemical/ biological assay employed (41).

Although the FPG test is a relatively inexpensive and standardized tool it is limiting since it measures a once-off blood glucose reading. It thereby fails to indicate daily glycemic fluctuations and captures only a single aspect of glucose metabolism. By disregarding the post-prandial state, abnormal glucose tolerance (indicative of pre-diabetes) is discounted (41). Since the FPG test captures only a single "snapshot" of glucose metabolism at a particular time, it is subject to large intra-individual variability discrediting its reliability and reproducibility (42). In addition, pre-analytical stability of the sample, inconvenience and duration of the fast, and patient stress and activity levels may impede the accuracy of the test (41).

The OGTT has long been referred to as the "gold standard" of diabetes diagnosis (41) (43). Since it measures post-prandial glucose fluctuations it can assess impaired glucose tolerance making it an early marker of diabetes – a very attractive property (41). It is also a better predictor of diabetes-associated complications than FPG (33) (44). However, it is impractical since it requires an overnight fast and availability for more than 2 hours in order to complete the test (41). OGTT also exhibits the highest intra-individual variability of all the currently available diagnostic tests (42), together with distinct gender differences that further impede its reliability (45).

Although HbA1c measures average glucose concentrations and does not require an overnight fast, it is subject to various other limitations (40). For example, the cost of the HbA1c assay is greater than the OGTT and FPG test which limits its global popularity and consequent standardization. Although accredited with exceptional specificity it lacks sensitivity and is thus not as accurate in diagnosing pre-diabetes as glucose-based methods which reflect the variance of glycemia and not merely the mean (36) (40). It is also not recommended as a diagnostic tool in a number of clinical conditions (40), where age, ethnicity (46), iron deficiency/ overload (47), and HIV-AIDS (48) may impact on HbA1c levels. Indeed, a study assessing the value of HbA1c in a South African setting established that it is not a viable diagnostic tool in this case (49).

1.3.3 The Future

Given the current status of diabetes diagnosis and the controversies discussed above, it is evident that investigations into novel diagnostic tools are justified. Here the conception of a new diagnostic tool, subject to fewer limitations and greater sensitivity, to detect the early onset of type 2 diabetes should be a valuable clinical advance.

1.4 ETIOLOGY

1.4.1 Is it as simple as “eat less, move more”?

While there exists a robust link between obesity and the development of insulin resistance/ type 2 diabetes, individuals with a wide range of body weights can and do develop diabetes (12) (50). For example, in many Asian countries where the prevalence of obesity is relatively low there is a surprisingly high incidence of type 2 diabetes (51). Obesity is also confounded by environmental, ethnic, socio-economic, behavioural, and genetic factors (51-54). Thus

although obesity remains a strong driving force underlying the development of insulin resistance and type 2 diabetes, it is not the only risk factor (55). Together these findings show that the development of type 2 diabetes is multi-faceted and subject to complex interactions (see Figure 1.2).

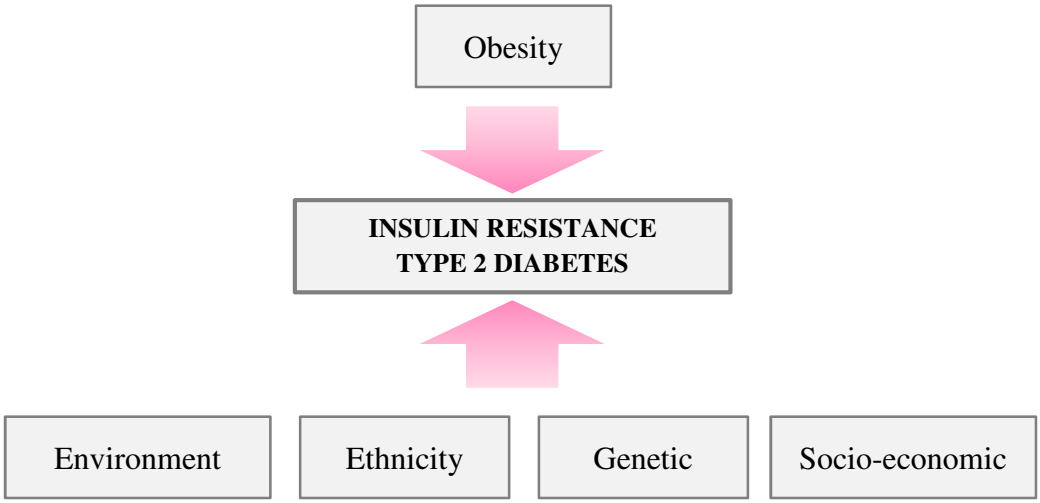

Figure 1. 2. The development of insulin resistance/ type 2 diabetes is multi-factorial.

1.4.1.1 Environment

While certain environmental factors such as access to a high caloric diet contribute directly to the development of obesity and thus insulin resistance/ type 2 diabetes, additional factors are independently linked to the development of diabetes (56). For example, industrialization has resulted in the promotion of a sedentary lifestyle and a global decrease in physical activity that is independently linked to the development and progression of diabetes (56) (57). More recently the direct association of environmental agents such as nicotine and certain pollutants with the development of type 2 diabetes are receiving increased attention (52).

1.4.1.2 Ethnicity

A number of epidemiological studies have shown that the risk of developing diabetes differs among different racial and ethnic groups, regardless of obesity (58-60). It is well recognized that Asians, particularly Asian Indians, who exhibit a relatively low prevalence of obesity present with an unexpectedly high incidence of type 2 diabetes (15) (51). This has been attributed to having a high abdominal adiposity distribution – an established risk factor for the development of type 2 diabetes (61). Accordingly, an individual may be of normal weight (defined by BMI), but simultaneously display a "metabolically-obese" phenotype resulting in insulin resistance (50) (62). Ethnicity therefore further adds to the complexity of developing insulin resistance/ type 2 diabetes independent of obesity (defined by BMI) through the concept of the metabolically obese, normal weight (MONW) person.

1.4.1.3 Socio-economic

A lower socio-economic status (SES) has long been linked to an increase in the prevalence of type 2 diabetes as well as various other health risks (53). While the link is often attributed to an increase in exposure to diabetes-associated risk factors such as decreased healthcare, obesity (cheap, carbohydrate- and fat-rich foods which are nutrient-deprived) and physical inactivity, all fuelled by poverty (53), a new relationship between SES and type 2 diabetes is emerging, namely chronic inflammation. Here scientists demonstrated that chronic inflammation, in response to stress, accounted for up to one third of the association between socio-economic disadvantage and the development of type 2 diabetes (63). Thus while a decrease in SES may expose individuals to environmental factors/ behavioural choices that may promote the development of obesity and consequently type 2 diabetes, SES may also be a direct cause of diabetes development through its association with chronic inflammation.

1.4.1.4 Behavioural

The consumption of cheap, nutrient-deprived, carbohydrate- and fat-rich foods is often necessary owing to poverty and decreased SES (53). However, many individuals who are not exposed to these circumstances continue to make unhealthy lifestyle choices despite knowing and understanding the risk factors associated with type 2 diabetes.

1.4.1.5 Genetic

In addition to the environmental evidence presented above, there is compelling evidence for the role of genetics in the pathogenesis of type 2 diabetes (64). The strong genetic component is substantiated by the fact that 40% of first degree relatives of patients suffering from diabetes will develop the disease themselves, when compared to 6% for the general population (54). Studies on monozygotic twins have further confirmed the role of genetics in the development of type 2 diabetes, i.e. the concordance rate of developing diabetes between monozygotic twins (~70%) is substantially higher than between dizygotic twins (~20%) (65) (66).

Additional genetic evidence stems from the high prevalence of diabetes among certain populations such as the Asian Indians and Mexican Americans (51, 67). Furthermore, monogenic cases of diabetes exist resulting in maturity-onset diabetes of the young (MODY) – a clinical subtype of type 2 diabetes accounting for 1-2% of total cases (68). While six individual mutations are described to cause MODY (69), polygenic cases of type 2 diabetes are far more complex. Since the completion of the human genome project (70), large scale genome-wide association studies (GWAS) are being carried out to in order to successfully link over 40 loci to the development of type 2 diabetes (71). Despite these successful attempts they only account for ~5 - 10% of the observed heritability associated with diabetes (54).

Thus a considerable genetic "dark-matter" still needs to be accounted for (64). Indeed, other biological explanations for disease susceptibility such as epigenetics (phenotypic variation without a change in the primary DNA sequence) and its effect on gene expression also need to be considered and assessed in order to obtain a better understanding of the risk for developing type 2 diabetes and elucidating the associated heritability (54). Moreover, changes in response to type 2 diabetes could aid in the identification of molecular markers that may prove valuable in tracking the state and progression of the disease (54) (72). Evidently, genetics plays a large but confounding role in the development of type 2 diabetes and the susceptibility loci identified thus far merely scrape the surface in the search for the missing heritability in type 2 diabetes (64). In addition to the independent contribution of genetics to type 2 diabetes, established links between genetics and the predisposition to developing obesity likewise exist (54) (73).

Taken together, it is clear that the development of type 2 diabetes is multi-faceted. Ultimately obesity remains a robust driving force behind the increasing prevalence of type 2 diabetes. For this reason it is important to understand that the simple "eat less, move more" mantra associated with obesity may be a gross oversimplification of a complex metabolic disease. As presented above there are numerous confounding factors that contribute to the development of obesity, demonstrating that the struggle against weight gain and consequent type 2 diabetes is a complicated and personal matter. Moreover, the body's ability to store excess energy, that has promoted the survival of our species, is no longer a necessity due to unlimited access to a high caloric diet. Consequently our bodies have not had enough time to genetically/ metabolically adapt to this "free food" diet further fuelling the obesity epidemic (Figure 1.3) (74).

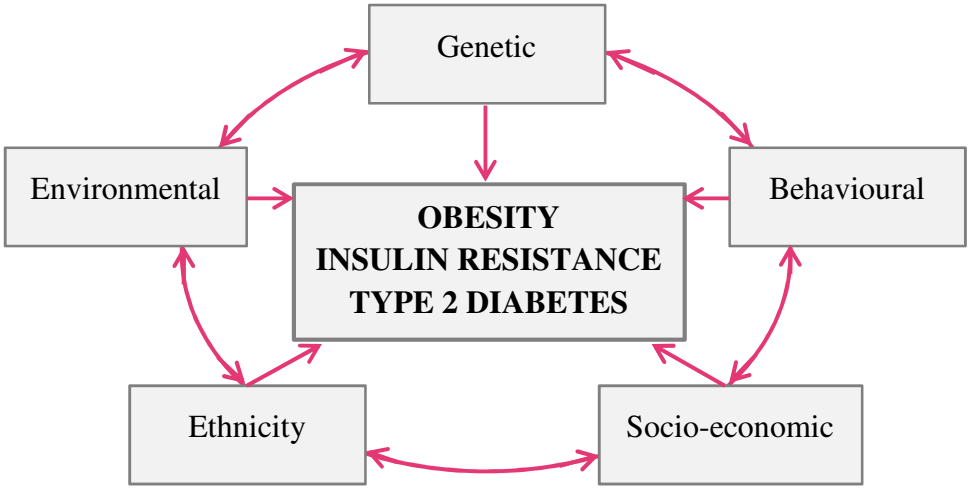

Figure 1. 3. The etiology of type 2 diabetes defies explanation by a single underlying causative agent.

1.4.2 Metabolic derangements associated with type 2 diabetes

It is widely acknowledged that the pathogenesis of type 2 diabetes is not entirely understood (69). Despite this many cell culture and animal models have been designed to increase our understanding of the progression and development of the disease, each replicating a set of genetic and metabolic changes that occur with type 2 diabetes (69) (75). The two most common forms of diabetes are type 1 diabetes (decreased insulin production) and type 2 diabetes (decreased response to insulin), although other rarer cases do exist such gestational diabetes and mono-genic cases (76). Type 2 diabetes accounts for 90 - 95% of all diabetes cases worldwide and is characterized by diminished insulin signaling and/ or insulin secretion with consequent hyperglycemia (19) (69).

1.4.2.1 Insulin resistance and hyperinsulinemia

Under normal physiological conditions insulin is released from pancreatic β-cells in response to a rise in blood glucose concentrations (e.g. with caloric intake) (77). Insulin subsequently lowers blood glucose concentrations by 1) increasing glucose uptake in insulin-dependent tissues (adipose, skeletal and hepatic) and 2) inhibiting glucose output by the liver (gluconeogenesis) and adipocytes (lipolysis), respectively (78). Maintaining glucose homeostasis is vital as increased glucose concentrations are harmful to many tissues while too low a decrease may result in hypoglycemia and associated complications e.g. comas (79). The key metabolic disturbances that occur with type 2 diabetes include: impaired insulin signaling; reduced insulin secretion; increased hepatic glucose output/ decreased glycogen synthesis and; increased FFA metabolism (lipolysis)/ decreased lipogenesis, although the precise underling molecular mechanisms are not yet well defined (69).

In brief, insulin signaling commences when insulin binds to the insulin receptor (IR) and consequently phosphorylates a host of substrates including insulin receptor substrate 1 (IRS1). IRS1 then binds phosphoinositide 3-kinase (PI3K), resulting in the activation of pyruvate dehydrogenase kinase (PDK) through its association with phosphatidylinositol-3,4,5-triphosphate (PIP3). PDK subsequently activates AKT (protein kinase B) through phosphorylation, resulting in a number of downstream effects critical for maintaining glucose homeostasis (80) (see Figure. 1.4).

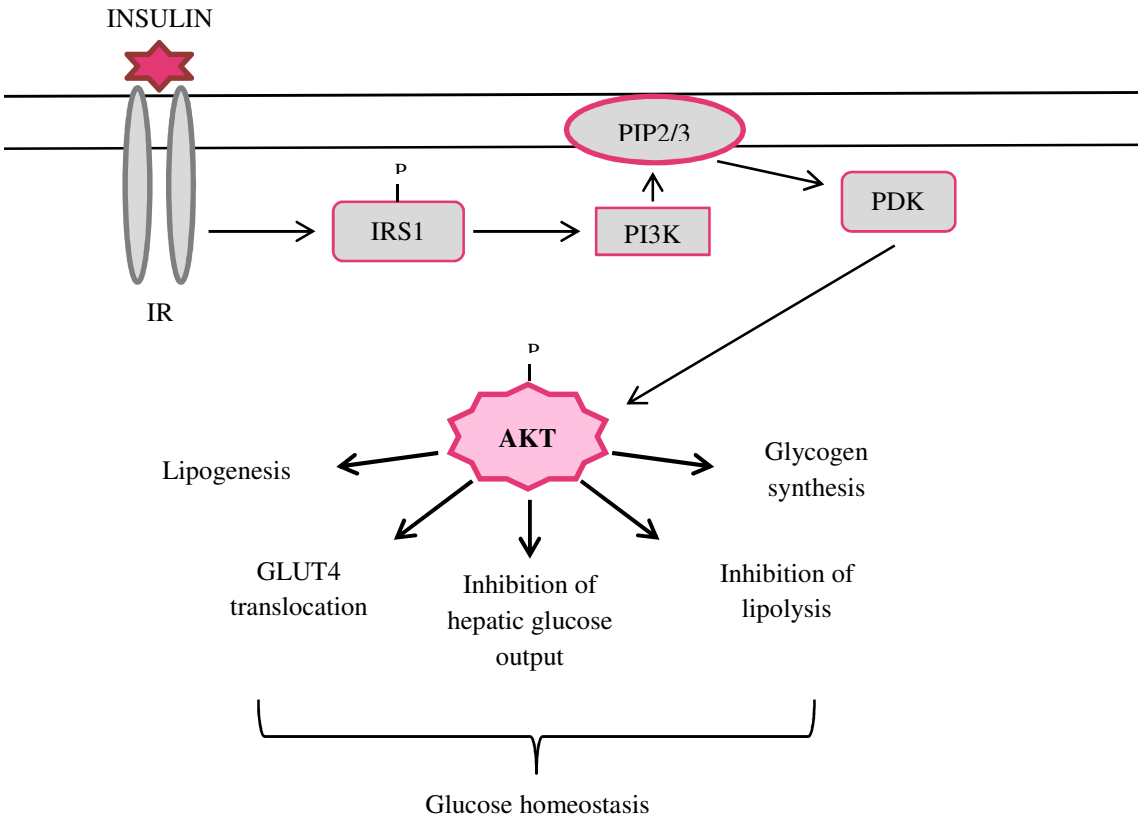

Figure 1. 4. Molecular mechanisms of insulin signaling and downstream effects on glucose metabolism.

GLUT4: Glucose transporter 4; IR: Insulin receptor; IRS1: Insulin receptor substrate 1; PDK: pyruvate dehydrogenase kinase; PIP2/ 3: phosphatidylinositol-3,4,5-triphosphate; PI3K: phosphoinositide 3-kinase.

Since glucose is a hydrophilic molecule, specific glucose transporters (GLUTs) are necessary for it to penetrate the lipid bilayer and enter the cell (81). AKT phosphorylation ultimately results in GLUT4 translocation to the sarcolemma for subsequent glucose uptake into the cell (reviewed in (82)). In addition, AKT phosphorylation further contributes to glucose homeostasis by controlling hepatic and lipid metabolism by way of influencing glycogen and lipid synthesis as well as preventing hepatic glucose output and lipolysis.

1.4.2.2 Impaired insulin signaling

Since insulin signaling involves a series of signaling cascades it may be impaired at many points in signal transduction. If insulin signaling is activated by phosphorylation it can likewise be inhibited by dephosphorylation. A number of inhibitory phosphatases act on the IR and other key modulators of insulin signaling including: SH2-containing tyrosine-protein phosphatase (SHP2); phosphatase and tensin homolog 10 (PTEN); protein-tyrosine phosphatase 1B (PTP-1B); and suppressor of cytokine signaling 3 (SOCS3), resulting in attenuated insulin signaling (82-85) (Figure 1.5). Furthermore, insulin signaling may also be diminished by inhibitory serine phosphorylation of IRS1, thereby inhibiting the IRS1/ PI3K complex and subsequent activation of AKT (80) (86) (Figure 1.5). Additionally, increased FFA metabolism results in impaired insulin signaling (12) (see section *1.4.2.3* for mechanism).

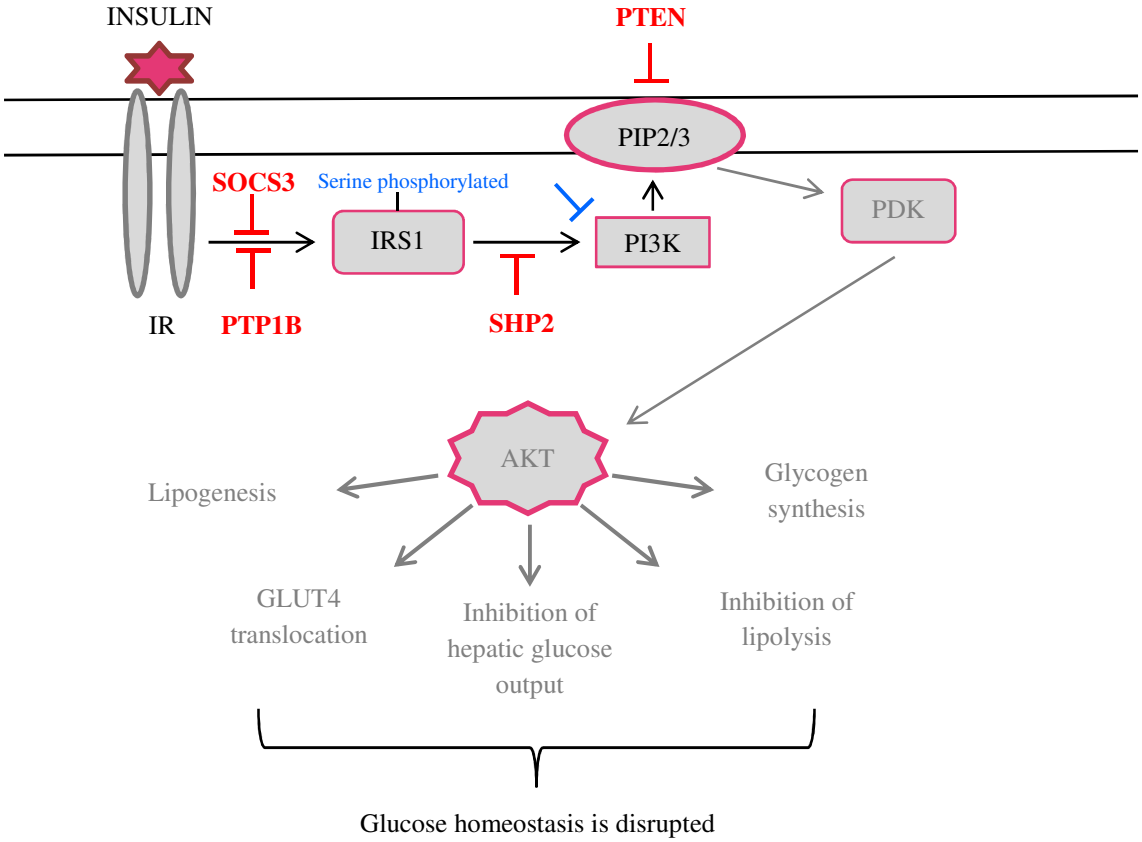

Figure 1. 5. Dephosphorylation results in impaired insulin signaling and disrupts glucose homeostasis.

A number of inhibitory phosphatases are able to modulate insulin signaling at various points in signal transduction (red), while IRS1 serine phosphorylation attenuates insulin signaling by preventing IRS1/ PI3K formation (blue).

GLUT4: Glucose transporter 4; IR: Insulin receptor; IRS1: Insulin receptor substrate 1; PDK: pyruvate dehydrogenase kinase; PIP2/ 3: phosphatidylinositol-3,4,5-triphosphate; PI3K: phosphoinositide 3-kinase; PTEN: phosphatase and tensin homolog; PTP1B: protein-tyrosine phosphatase 1B; SHP2: SH2-containing tyrosine-protein phosphatase; SOCS3: suppressor of cytokine signaling 3

1.4.2.3 Increased hepatic glucose output/ decreased glycogen synthesis

During times of nutrient excess, AKT is phosphorylated and consequently inhibits glycogen synthase kinase 3 (GSK3) which in turn prevents it from inhibiting its substrate, glycogen synthase, promoting glycogen synthesis and storage (87) (Figure 1.6 A). However, when insulin signaling is diminished AKT is not activated and hence glycogen synthesis is not stimulated (87) (Figure 1.6 B). This results in elevated blood glucose concentrations since excess glucose is consequently not stored as glycogen. AKT activation further contributes to

hepatic glucose metabolism since it results in a decrease in transcription of two gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) through the phosphorylation of forkhead transcription factor box protein (FOXO) (80) (87) (88) (Figure 1.6 A). As a result, it limits the production of glucose through gluconeogenesis (88) (89). Hence when signaling is disrupted, hepatic glucose output is increased which further augments hyperglycemia (88) (Figure 1.6 B).

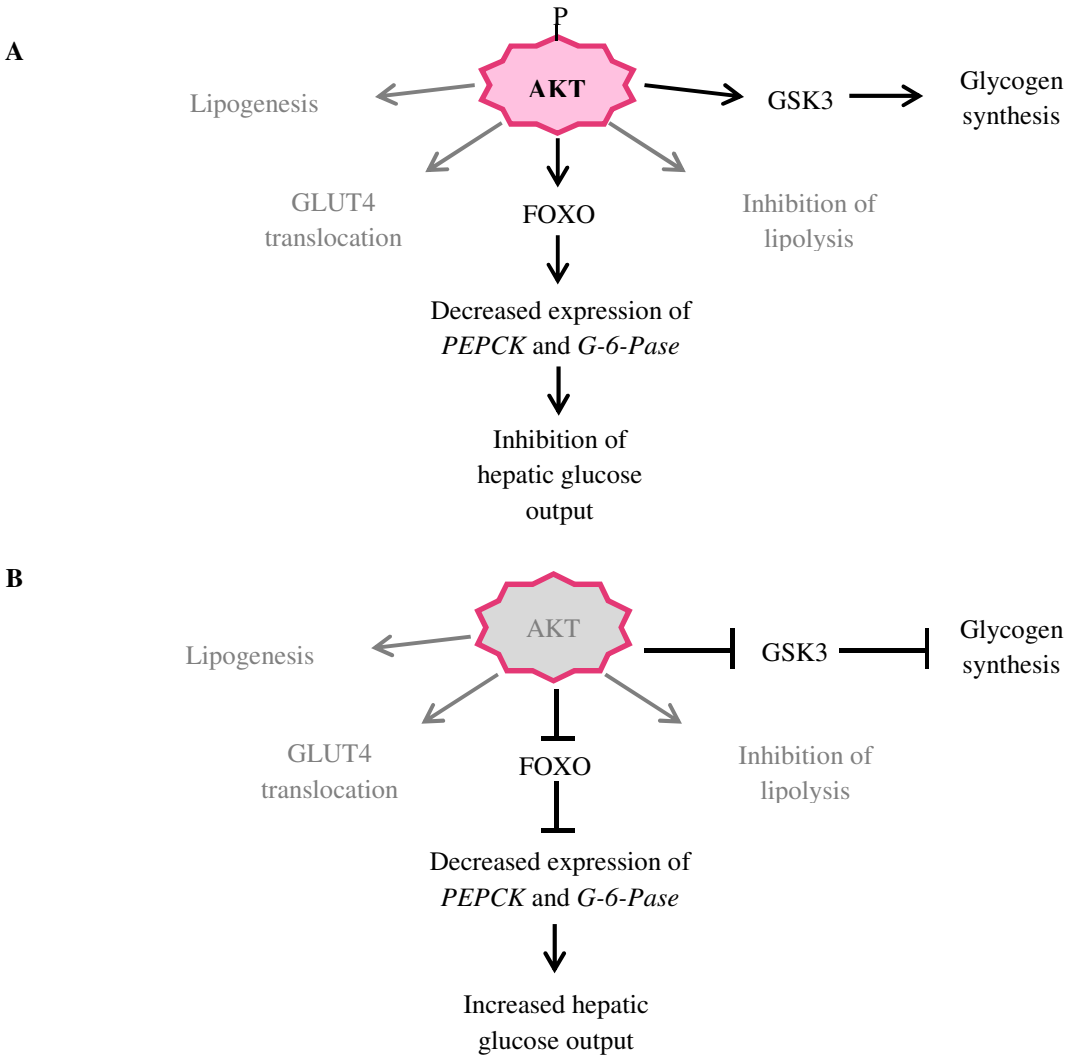

Figure 1. 6 A: Phosphorylated AKT promotes glycogen synthesis and inhibits hepatic glucose output. B: Impaired insulin signaling results in the inhibition of glycogen synthesis and promotes hepatic glucose output, thereby augmenting hyperglycemia.

A: Under normal physiological conditions AKT is phosphorylated (upon insulin stimulation) and consequently regulates hepatic glucose metabolism by: 1) promoting glycogen synthesis via the inhibition of GSK3 with subsequent promotion of glycogen synthase and 2) decreasing hepatic glucose output by phosphorylating FOXO and lowering *PEPCK* and *G-6-Pase* transcription.

B: Diminished AKT phosphorylation promotes hyperglycemia by decreasing glycogen synthesis via the inhibition of the enzyme glycogen synthase and increases hepatic glucose output via reduced FOXO phosphorylation with consequent rises in *PEPCK* and *G-6-Pase* transcription.

AKT: Protein kinase B; FOXO: Forkhead transcription factor box protein; GLUT4: Glucose transporter 4; GSK3: Glycogen synthase kinase 3; G-6-Pase: Glucose-6-phosphatase; PEPCK: phosphoenolpyruvate carboxykinase.

1.4.2.4 Increased fatty acid metabolism/ decreased lipogenesis

AKT phosphorylation regulates lipid metabolism by inhibiting lipolysis and promoting the storage of glucose as triglycerides through enhanced lipid production (87). Phosphorylated AKT promotes lipid biosynthesis through promoting the stability of sterol regulatory element-binding proteins (SREBPs) which are transcription factors responsible for the upregulation of genes involved in fatty acid synthesis (87). Thus when insulin signaling is impaired AKT fails to enhance SREBP stability, thereby inhibiting fatty acid synthesis (87). It is also proposed that AKT activation can inhibit lipolysis through the activation of phosphodiesterase 3 B (*PDE3B*) – a gene suggested to inhibit the breakdown of fatty acids (90) (91).

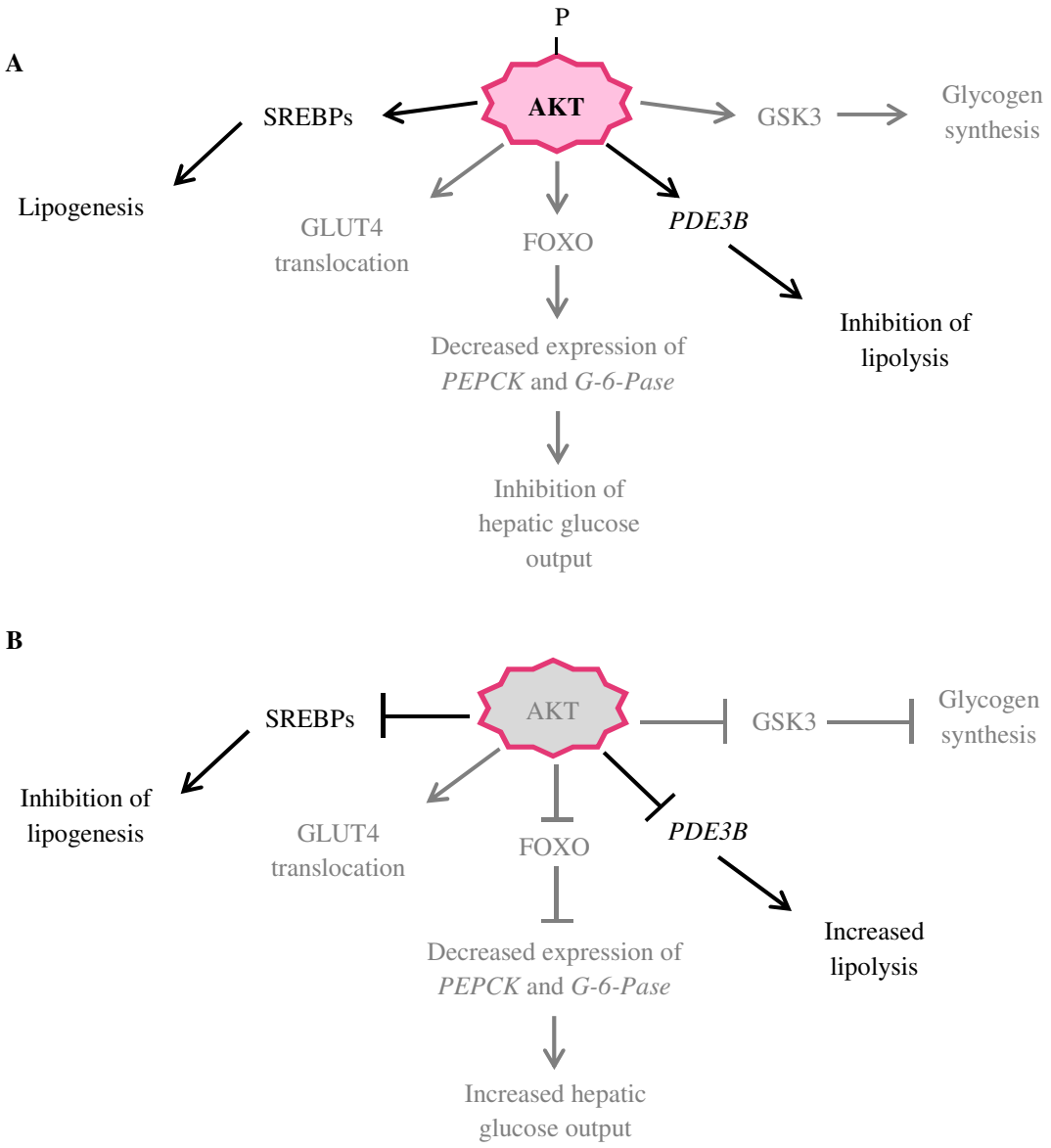

Figure 1. 7 A: Phosphorylated AKT promotes fatty acid synthesis and inhibits lipolysis. B: Attenuated insulin signaling results in the inhibition of lipogenesis and promotes fatty acid metabolism, thereby augmenting hyperglycemia.

A: Upon insulin stimulation, phosphorylated AKT enhances fatty acid biosynthesis by inhibiting the breakdown of lipids by activating *PDE3B* and promoting the formation of lipids by increasing SREBP stability.

B: Attenuated AKT phosphorylation prevents lipid synthesis by decreasing the stability of SREBPs while concurrently increasing the breakdown of lipids (via *PDE3B* inhibition), promoting FFA metabolism.

AKT: Protein kinase B; FOXO: Forkhead transcription factor box protein; GLUT4: Glucose transporter 4; GSK3: Glycogen synthase kinase 3; G-6-Pase: Glucose-6-phosphatase; PEPCK: phosphoenolpyruvate carboxykinase; PDE3B: Phosphodiesterase 3 B; SREBPs: Sterol regulatory element-binding proteins.

Enhanced fatty acid metabolism refers to the increased metabolism of non-esterified fatty acids (NEFAs) for energy production and may be the single most important factor contributing to the loss of insulin sensitivity with type 2 diabetes (12). Since there is a consequential increase in adipocyte mass and number with the development of obesity/ type 2 diabetes there is a resultant increase in NEFA metabolism (12) (92). Indeed, individuals suffering from type 2 diabetes present with increased circulating NEFAs (93). The role of NEFAs in insulin resistance was first described exactly 50 years ago by Randle et al. (94). Here it was proposed, through the glucose-fatty acid cycle, that glucose oxidation was inhibited due to an increase in free fatty acids (FFAs). Increased FFA oxidation results in greater acetyl-CoA/CoA and NADH/NAD^+ ratios in the mitochondrion. This in turn inhibits pyruvate dehydrogenase (PDH) activity causing an increase in cytosolic citrate and a consequent decrease in phosphofructokinase (PFK) levels – a key glycolytic enzyme. Consequently, upstream glycolytic intermediates such as glucose-6-phosphate (G-6-P) accumulate, eventually resulting in the inhibition of hexokinase and an accumulation of intracellular glucose and decreased glucose uptake through GLUT4 (94) (95).

It has since also been proposed that decreased glucose uptake rather than altered glucose metabolism (described by Randle) may be responsible for fatty acid-induced insulin resistance (78). Here metabolites of FFAs such as acyl-CoAs, ceramides, and diacylglycerol (DAG) are proposed to disrupt insulin signaling through the activation of protein kinase C (PKC) (Figure 1.8). PKC activation promotes the inhibitory serine phosphorylation of IRS1 thereby impeding the association of IRS1 with PI3K and ultimately decreasing AKT phosphorylation/ activation. The consequences include decreased GLUT4 translocation, increased hepatic glucose output and, lipolysis (discussed and explained in sections *1.4.2.1 - 1.4.2.4*) creating a vicious cycle of insulin resistance with consequent hyperglycemia.

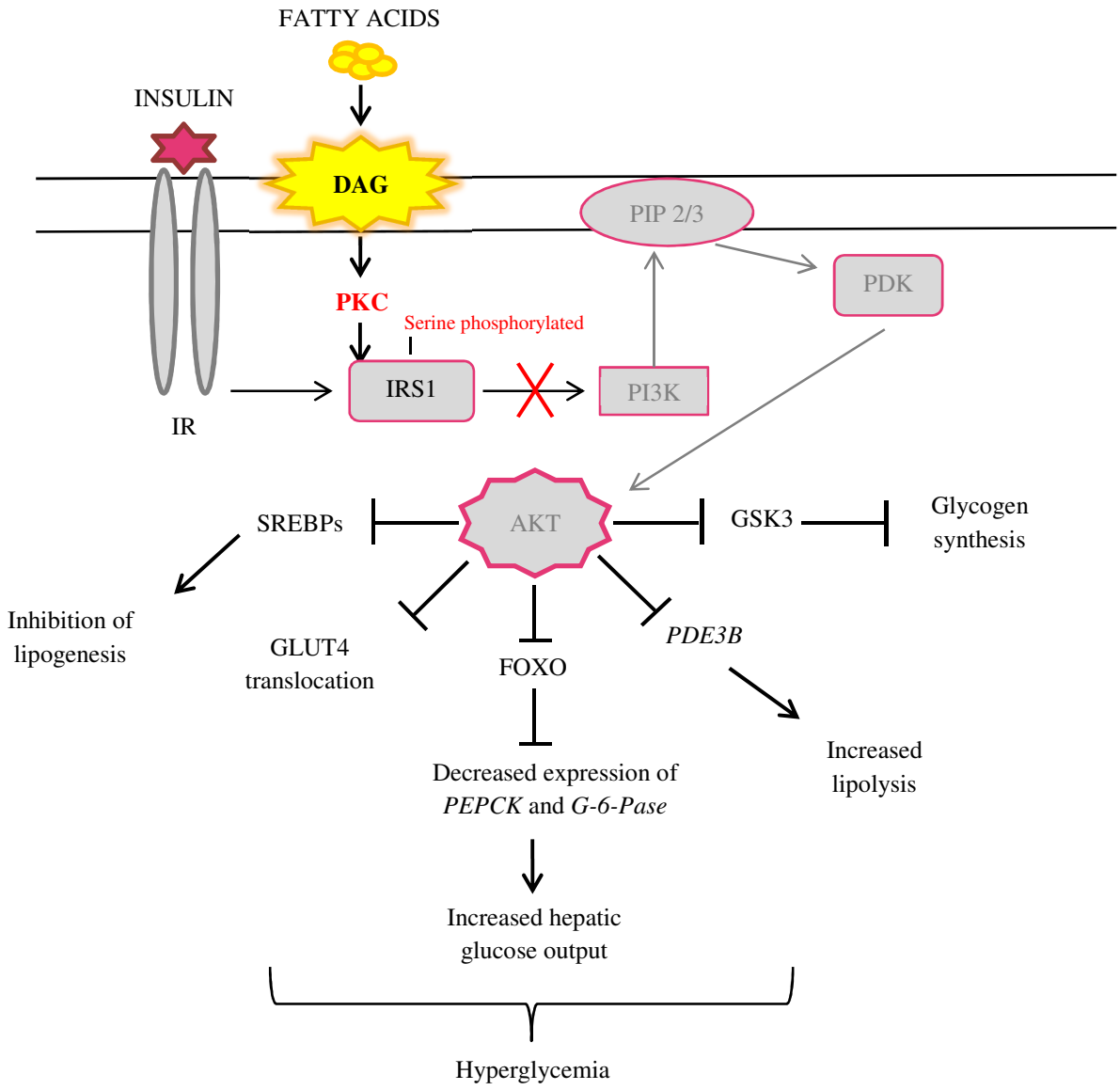

Figure 1. 8. FFAs release DAG which activates PKC and inhibits IRS1/ PI3K association, resulting in diminished insulin signaling.

FFA metabolism leads to the release of DAG which in turn activates PKC. PKC initiates a serine/ threonine cascade resulting in IRS1 serine phosphorylation. Since the association of IRS1 and PI3K is dependent on threonine phosphorylation the complex is inhibited resulting in diminished insulin signaling.

AKT: Protein kinase B; DAG: Diacylglycerol; FOXO: Forkhead transcription factor box protein; GLUT4: Glucose transporter 4; GSK3: Glycogen synthase kinase 3; G-6-Pase: Glucose-6-phosphatase; IR: Insulin receptor; IRS1: Insulin receptor substrate 1; PEPCK: phosphoenolpyruvate carboxykinase; PDK: pyruvate dehydrogenase kinase; PDE3B: Phosphodiesterase 3 B; PIP2/ 3: phosphatidylinositol-3,4,5-triphosphate; PI3K: phosphoinositide 3-kinase; PTEN: phosphatase and tensin homolog; PTP1B: protein-tyrosine phosphatase 1B; SHP2: SH2-containing tyrosine-protein phosphatase; SOCS3: suppressor of cytokine signaling 3; SREBPs: Sterol regulatory element-binding proteins.

While the principal function of the adipocyte is to store excess energy in the form of triglycerides and release FFA molecules into circulation as needed, it also fulfils an additional important function (77). Here adipocytes release certain hormones and cytokines that play a central role in the regulation of insulin signaling (reviewed in (96)).

1.4.2.5 Diminished insulin secretion

Lower insulin secretion is another key pathophysiological event contributing towards the development of type 2 diabetes (80). Insulin resistance and subsequent hyperinsulinemia may precede the development of hyperglycemia by years or even decades (97). The pancreatic β-cells are initially able to compensate for the loss of insulin sensitivity by increasing insulin production (12) (98). This in turn allows the body to maintain a blood glucose level within the normal, healthy range allowing for a normoglycemic, but hyperinsulinemic state (99). As the disease progresses and insulin resistance rises, the large amounts of insulin produced by the pancreas are no longer able to maintain glucose homeostasis and blood glucose levels rise (100). This results in pre-diabetes, initially characterized by mild hyperglycemia in combination with hyperinsulinemia (12) (Figure 1.9). This prolonged state of hyperinsulinemia in combination with gluco-lipotoxicity becomes detrimental to β-cell function and subsequently insulin levels begin to decline (77). As insulin production decreases and insulin sensitivity is further blunted, blood glucose levels are elevated into the diabetic range with simultaneous decreases in insulin concentrations (100) (Figure 1.9). Eventually the β-cells become completely exhausted and those patients with long established full-blown diabetes become dependent on exogenous supplies of insulin in order to maintain normal glucose homeostasis (101) (102).

Additional causes of insulin resistance include: mutations in the IRS1 protein; severe mitochondrial dysfunction as seen with aging; and attenuated IR gene expression (80).

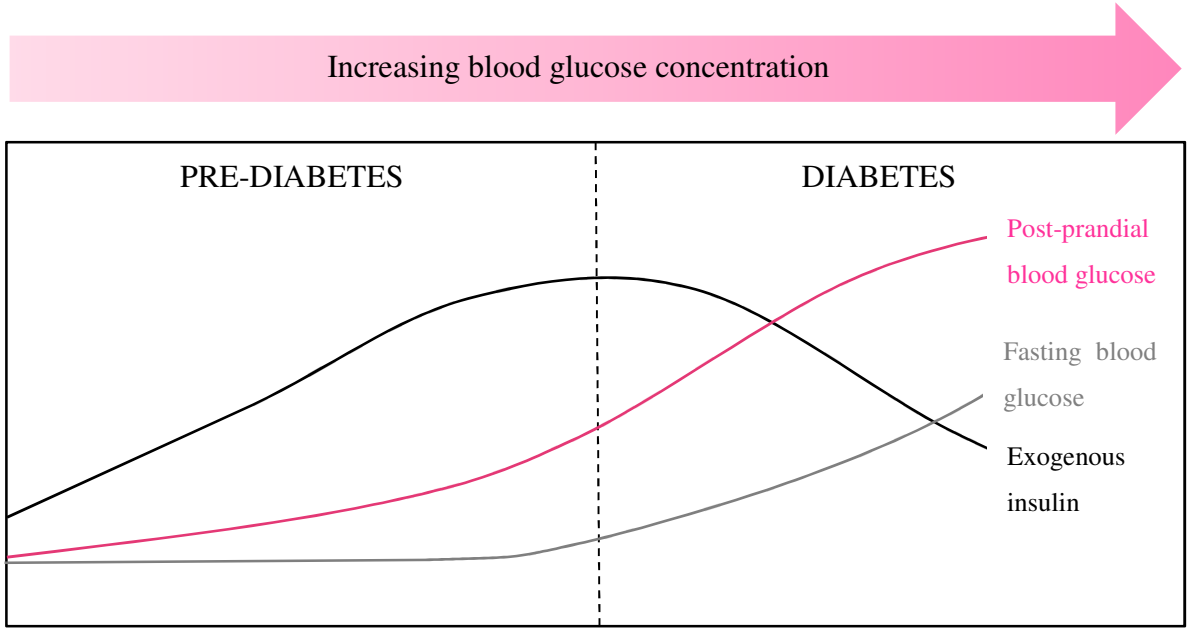

Figure 1. 9. The natural biochemical progression of type 2 diabetes.

Adapted from (103).

Briefly, insulin resistance in adipocytes, skeletal muscle and the liver contribute to the development and progression of hyperglycemia and consequent type 2 diabetes (69). A decrease in adipocyte insulin sensitivity prevents the inhibition of lipolysis thereby increasing circulating FFAs and subsequently promoting insulin resistance (12) (78) (94) (95). Hepatic insulin resistance prevents the storage of glucose as glycogen, and promotes gluconeogenesis thereby increasing hepatic glucose output resulting in augmented hyperglycemia (80). Skeletal muscle is the major site of glucose disposal (~75%) thereby making skeletal muscle insulin resistance a primary contributing factor to the pathogenesis of type 2 diabetes (104).

1.4.2.6 Hyperglycemia

Both insulin-dependent (described above) as well as insulin-independent mechanisms regulate the uptake of glucose into cells (81). Since glucose is a hydrophilic molecule, specific glucose transporters are necessary for glucose to penetrate the lipid bilayer and enter the cell (81). As GLUT4 translocation is diminished with insulin resistance/ type 2 diabetes there is a consequent global decrease in glucose uptake by insulin-dependent tissues resulting in elevated blood glucose concentrations (81). Surplus glucose then enters insulin-independent tissues by facilitated diffusion, down a concentration gradient (high concentration of glucose in the blood *vs.* low concentration of glucose in the insulin-independent tissues) causing elevated glucose flux in these tissues (81). A growing body of evidence suggests that augmented glucose metabolism promotes oxidative stress production, initiating downstream metabolic derangements (105).

1.5 HYPERGLYCEMIA INDUCES OXIDATIVE STRESS

Progressive hyperglycemia is widely accepted as the single most important factor contributing to micro- and macrovascular pathologies associated with type 2 diabetes (106). A unifying hypothesis has identified hyperglycemia-induced production of reactive oxygen species (ROS) as the primary mechanism behind the associated tissue damage (106). ROS are products of normal cellular metabolism and carry out a variety of functions under physiological conditions (107). However, with chronic hyperglycemia homeostasis is disturbed and the balance between ROS production and its scavenging is disrupted (107). Augmented glucose metabolism concurrently increases the production of ROS and impairs a number of antioxidant defence mechanisms, resulting in a global increase in oxidative stress

(107). An abundance of ROS leads to protein, lipid and DNA damage (108), culminating in physiological dysfunction and a host of pathologies including diabetes.

1.5.1 Mitochondrial superoxide production is the primary source of ROS

The mitochondrion has been identified as the primary source of ROS production during hyperglycemia through the uncoupling of the mitochondrial electron transport chain (ETC) (reviewed in (79)). Under physiological conditions the body generates energy in the form of ATP through the oxidation of pyruvate. Pyruvate enters the tricarboxcylic acid cycle (TCA) where NADH and $FADH_2$ are produced in addition to CO_2. These reducing equivalents subsequently enter the oxidative phosphorylation pathway/ mitochondrial ETC to generate ATP. Normally, electron transfer occurs at complexes I, III, and IV moving protons outward into the intermembrane space, creating a proton gradient (109). This results in protons passively moving back through the mitochondrial inner membrane space into the matrix, driving ATP synthase (situated at complex IV) and hence mitochondrial ATP generation (109). ROS (in the form of mitochondrial superoxide [$O_2^{\bullet -}$]) production occurs naturally at complexes I and III of the ETC, although complex II has also recently been implicated in $O_2^{\bullet -}$ production (109, 110). Here antioxidants such as superoxide dismutase (SOD) degrade volatile oxygen free radicals to hydrogen peroxide (H_2O_2) that is then converted to water and oxygen by catalase (107).

However, cells containing high intracellular glucose levels (e.g. diabetic individuals) have higher levels of glucose-derived pyruvate passing though the TCA cycle (109). Hence more NADH and $FADH_2$ are produced and shunted into mitochondrial ETC resulting in a higher concentration of electron donors that increases the gradient potential across the mitochondrial

membrane (109). Once a critical threshold is reached, electron transfer at complex III is blocked triggering electrons to accumulate at coenzyme Q (CoQ) (111). CoQ then begins to donate electrons to molecular oxygen resulting in the formation of superoxide (109). Since hyperglycemia concurrently increases ROS production and inhibits antioxidant defence mechanisms, the excess superoxide is subsequently not converted into less harmful by-products (107).

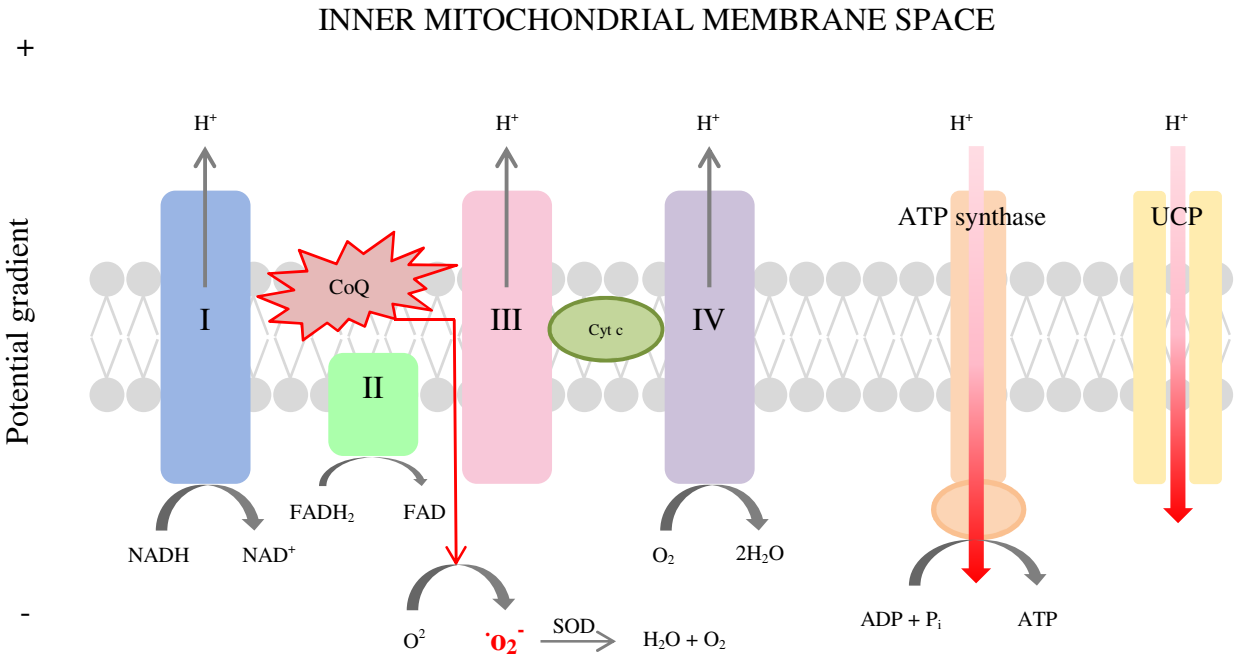

Figure 1. 10. Hyperglycemia induces oxidative stress via the uncoupling of the mitochondrial electron transport chain.

Adapted with permission from (109).

During times of hyperglycemia, increased glycolysis results in elevated levels of electron donors (NADH and $FADH_2$) entering the mitochondrial electron transport chain. This increases the gradient potential across the mitochondrial membrane resulting in defective electron transfer and eventual superoxide production.

ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CoQ: Coenzyme Q; Cyt C: Cytochrome C; FAD: Flavin adenine dinucleotide (oxidized); FADH2: Flavin adenine dinucleotide (reduced); H_2O: Water; NAD+ :Nicotinamide adenine dinucleotide (oxidized); NADH: Nicotinamide adenine dinucleotide (reduced); O2•-:superoxide; SOD: Superoxide dismutase; UCPs: Uncoupling proteins.

Although superoxide is believed to be the primary source of ROS, other forms are also produced by the mitochondrion under hyperglycemic conditions (107). For example, H_2O_2

that is usually converted to water and oxygen by the enzyme catalase is inhibited under hyperglycemic conditions allowing H_2O_2 to be converted to various other ROS forms such as the hydroxyl ion (•OH) (112).

Hence with insulin resistance and subsequent chronic hyperglycemia, oxidative stress is produced via the uncoupling of the mitochondrial electron transport chain. Cells that are unable to control intracellular glucose levels, such as those susceptible to diabetic complications (113), are exposed to sustained hyperglycemic conditions and consequently develop increased levels of hyperglycemia-mediated ROS (114). Despite the intrinsic protective effects of antioxidants such as SOD, catalase, and reduced glutathione, an abundance of hyperglycemia-induced ROS persists and ultimately leads to micro- and macrovasulature problems that accompany type 2 diabetes (107) (114).

Although mitochondrial-mediated oxidative stress is the primary source of ROS production in the etiology of type 2 diabetes, additional sources of oxidative stress also exist such as the activation of alternative non-oxidative glucose-utilizing pathways (NOGPs) (to be discussed in detail below) and the production of nitric oxide (·NO) via the nitric oxide synthase pathway (107).

1.6 HYPERGLYCEMIA-INDUCED SUPEROXIDE PRODUCTION UPREGULATES ALTERNATIVE NON-OXIDATIVE GLUCOSE-UTILIZING PATHWAYS

While glycolysis utilizes the majority of glucose entering the cell, a small amount of glucose is metabolized via alternative NOGPs (106) (107). However, with hyperglycemia excess glucose is diverted into the alternative NOGPs resulting in their upregulation (106). Increased

flux through four of the five minor glycolytic pathways has been implicated in the development of diabetes-associated complications (105).

Hyperglycemia-induced activation of NOGPs is thought to be initiated by a single upstream metabolic event namely, the overproduction of mitochondrial superoxide (105) (106). Here the mechanism put forward is that mitochondrial superoxide induces DNA strand breakage resulting in the activation of poly (ADP-ribose) polymerase (PARP) – an enzyme responsible for DNA repair (105) (106). In addition to the role PARP plays in DNA repair it has the ability to inhibit the activity of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (115). GAPDH inhibition results in the accumulation of upstream glycolytic intermediates that are then diverted into the five alternative NOGPs, i.e. 1) the polyol pathway, 2) the pentose phosphate pathway (PPP), 3) the formation of advanced glycation end products (AGE), 4) activation of PKC and 5) the upregulation of the hexosamine biosynthetic pathway (HBP) (106) (Figure 1.11). Moreover, NOGPs may also be upregulated due to increased substrate (glucose) availability experienced with hyperglycemia.

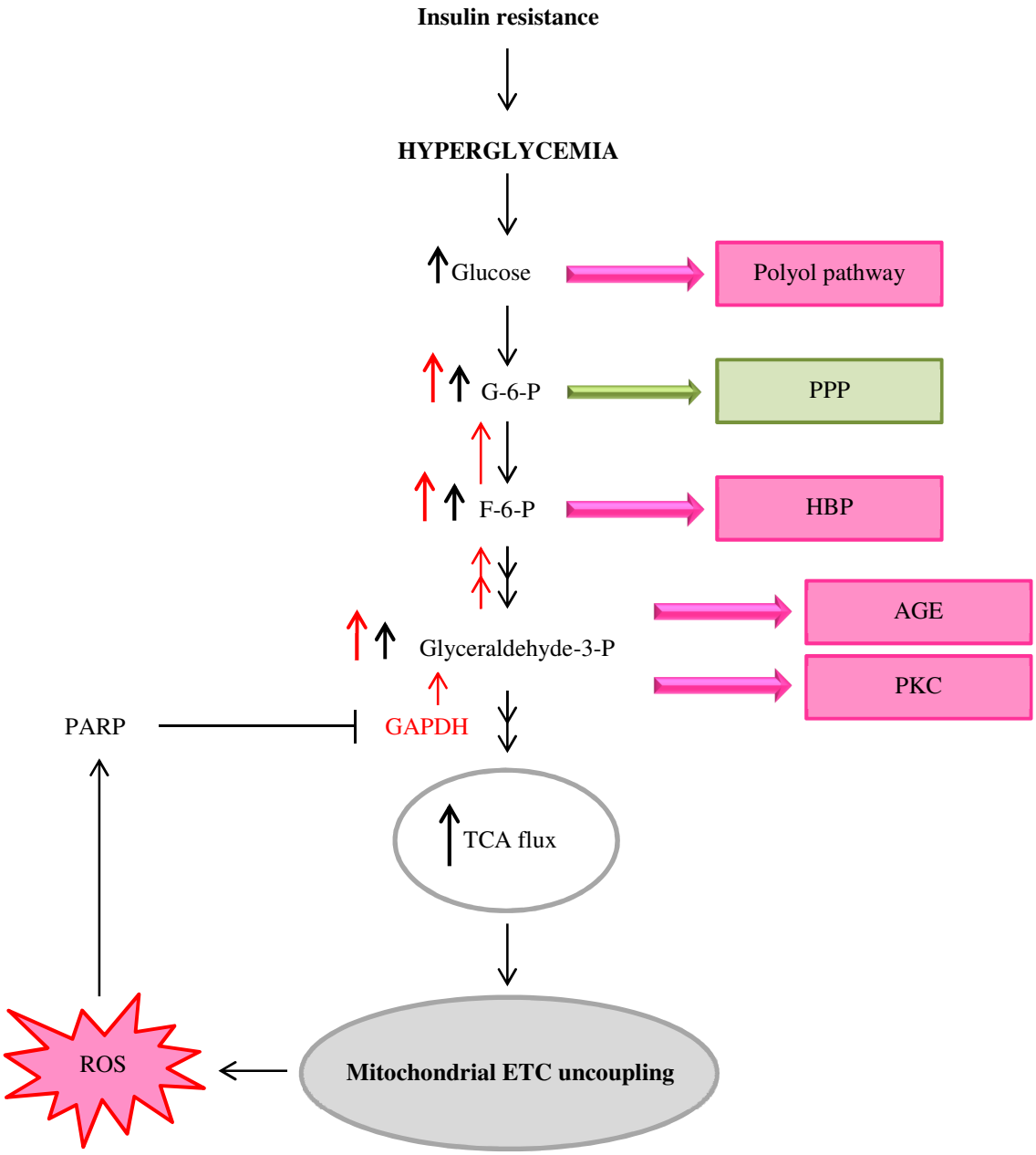

Figure 1. 11. Hyperglycemia-mediated superoxide production inhibits GAPDH activity resulting in the upregulation of alternative NOGPs.

→ Hyperglycemia augments glycolytic flux and increases mitochondrial ROS production. This activates PARP which subsequently inhibits GAPDH activity.→ GAPDH inhibition results in further accumulation of upstream glycolytic intermediates which are then diverted into the five alternative NOGPs.

AGE: Advanced glycation end products; ETC: Electron transport chain; F-6-P: Fructose-6-phosphate; G-6-P: Glucose-6-phosphate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HBP: Hexosamine biosynthetic pathway; PARP: Poly(ADP) polymerase; PPP: Pentose phosphate pathway; PKC: Protein kinase C; ROS: Reactive oxygen species; TCA: Tricarboxcylic acid cycle.

1.6.1 The Polyol Pathway

Under normal physiological conditions ~3% of total glucose is metabolized by the polyol pathway (112). This pathway is a two-part metabolic process where toxic aldehydes are converted to inactive alcohols and then further converted to fructose. The primary function of the polyol pathway is to reduce toxic aldehydes into inactive alcohols (116). The reduction is accomplished by a family of aldo-keto reductase enzymes with the aid of the reducing agent NADPH (116). Under normoglycemia aldose reductase (encoded by aldo-keto reductase family 1, member B1 [*AKR1B1*]) has a low affinity for glucose; however, during hyperglycemia increased intracellular glucose concentrations result in augmented polyol flux (109). This subsequently increases the reduction of toxic aldehydes to inactive alcohols resulting in decreased NADPH availability (106). However, NADPH is also required as a co-factor for the conversion of oxidized glutathione to reduced glutathione – a potent antioxidant (106) (107). Hence increased flux through the polyol pathway during times of hyperglycemia depletes NADPH and further contributes towards oxidative stress by inhibiting the formation of reduced glutathione, a potent antioxidant (109). Furthermore, the conversion of inactive alcohols (sorbitol) to fructose by sorbitol dehydrogenase may activate PKC through an increased NADPH/NAD^+ ratio, further exacerbating ROS production (107). (see section 1.6.2 for PKC activation) (refer back to Figure 1.8 for the mechanism of PKC activation and hyperglycemia).

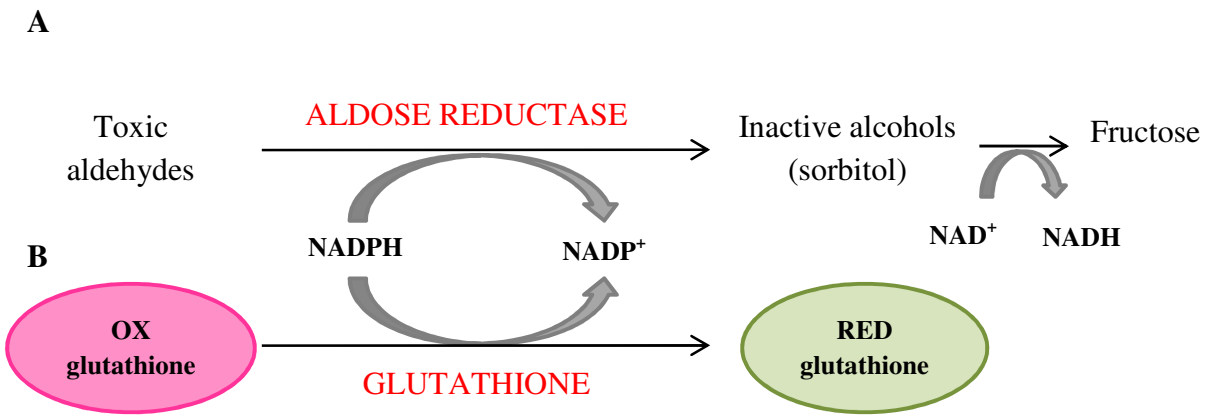

Figure 1. 12. Schematic representation of the polyol pathway.

A. Aldose reductase converts toxic aldehydes into inactive alcohols using NADPH as a cofactor. B. NADPH is an important cofactor in the conversion of oxidized glutathione to reduced glutathione.

NAD^+: Nicotinamide adenine dinucleotide (oxidized); NADH: Nicotinamide adenine dinucleotide (reduced); $NADP^+$: Nicotinamide adenine dinucleotide phosphatase (oxidized); NADPH: Nicotinamide adenine dinucleotide phosphatase (reduced); OX: Oxidized; RED: Reduced.

1.6.2 PKC activation

The PKC family is comprised of at least 11 known isoforms that play a fundamental role in serine/ threonine signaling cascades involved in insulin signaling transduction (117). Under hyperglycemic conditions enhanced flux through the PKC activation pathway results in an increase of the upstream intermediate, dihydroxyacetone phosphate, that is then reduced to α-glycerol-phosphate (118). When the latter is conjugated with fatty acids it promotes the formation of DAG that subsequently activates PKC (118) (Figure 1.13). PKC may also be activated directly by the stimulation of phospholipase C, by the inhibition of DAG kinase (112) (119) (Figure 1.13), indirectly by increased flux through the polyol pathway, or by altering the activity of AGEs and their receptors (120) (121). PKC has a detrimental effect on insulin signaling and promotes hyperglycemia that in turn contributes to enhanced ROS production (refer back to section *1.4.2.4*). Furthermore, since PKC promotes the activity of NADPH oxidase (thereby decreasing NADPH availability), greater PKC activity lowers the levels of reduced glutathione resulting in a reduction in antioxidant capacity (107) (Figure

1.13). The reduction in antioxidant capacity in combination with increased ROS production, via impaired insulin signaling with consequent hyperglycemia, further drives the cell into a state of increased oxidative stress. The over activity of PKC is also linked to harmful functional changes in vascular tissue (122-124).

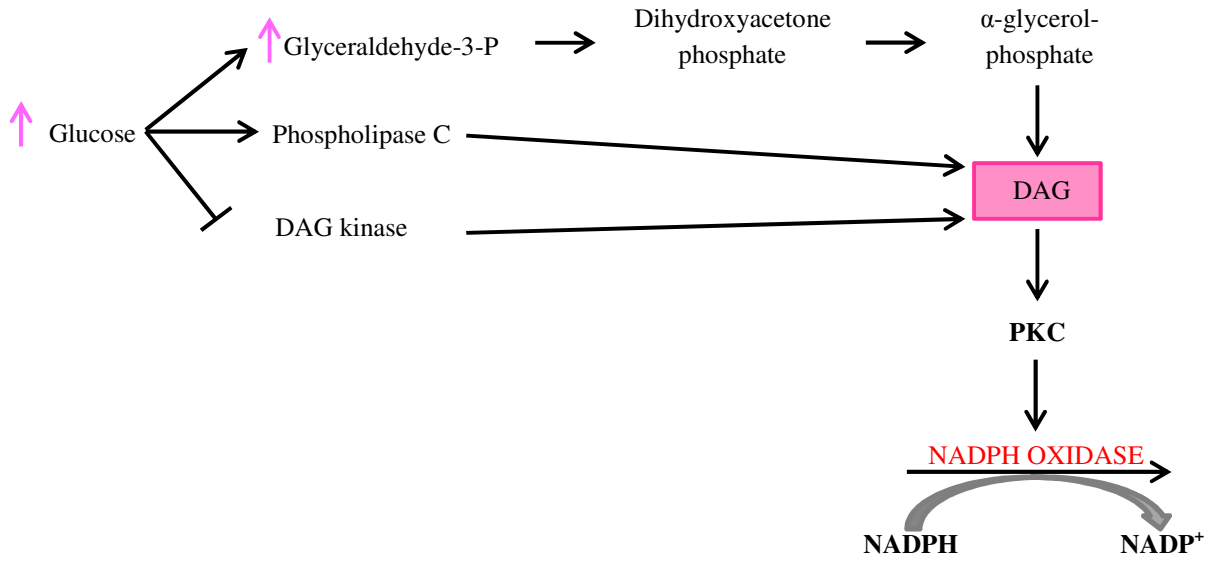

Figure 1. 13. Schematic representation of the PKC pathway and its activation.

DAG is formed by increases in glyceraldehyde-3-P, the activation of phospholipase C and the inhibition of DAG kinase. Together these mechanisms result in the activation of PKC which promotes NADPH oxidase activity and consequently decreases NADPH availability.

DAG: Diacylglycerol; Glyceraldehyde-3-P: Glyceraldehyde-3-Phosphate; PKC: Protein kinase C; NADPH: Nicotinamide adenine dinucleotide phosphatase (reduced); $NADP^+$: Nicotinamide adenine dinucleotide phosphatase (oxidized).

1.6.3 Formation of AGEs

Increased intracellular glucose initiates the formation of advanced glycation end products in three distinct ways (125). AGEs arise from: the auto-oxidation of glucose, decomposition of the Amadori product (an organic reaction resulting in the rearrangement of a glucose molecule), and the non-enzymatic reaction of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Figure 1.14) (125)

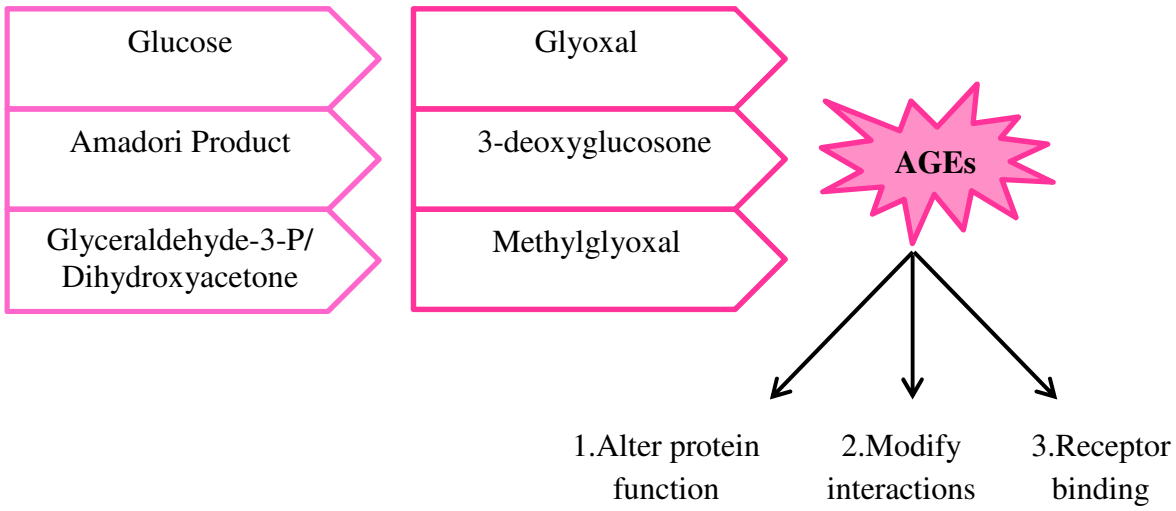

Figure 1. 14. The formation of AGEs and its harmful effects

AGEs: Advanced glycation end products; Glyceraldehyde-3-P: Glyceraldehyde-3-phosphate.

The products of the three above-mentioned reactions namely; glyoxal (from glucose), 3-deoxyglucosone (from Amadori product) and methylglyoxal (from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) then react with amino groups on intra- and extracellular proteins to form AGEs (112). The formation of AGE precursors are able to harm target cells by three common mechanisms: 1) intracellular proteins that are modified by AGEs exhibit altered function, 2) AGE precursors are able to modify extracellular matrix components of target proteins thereby modifying their interaction with other matrix

components of target proteins thereby altering its interaction with other matrix components and receptors, 3) proteins modified by AGE precursors result in its binding to respective receptors (e.g. RAGE) (126) (127). The latter results in the formation of ROS, that in turn activates the ubiquitous transcription modulator nuclear factor kappa B (NF-κB) thereby further contributing to ROS formation (126). Since AGEs are present in several tissues of diabetic patients its formation is considered a major contributor towards the onset of diabetic complications (112).

1.6.4 The Hexosamine Biosynthetic Pathway

Under normal physiological conditions a small percentage of intracellular glucose (2 - 5%) is diverted from glycolysis and shuttled into the HBP, culminating in the assembly of uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc) (128). Fructose-6-phosphate is diverted from the central glycolytic pathway and converted to glucosamine-6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT a.k.a. GFPT) (129). Glucosamine-6-phosphate is then converted – via a series of reactions – to UDP-GlcNAc, the end product of this pathway (129). UDP-GlcNAc molecules are able to attach to serine/ threonine residues on target proteins resulting in post-translational modifications (PTMs) (130). This process is commonly known as *O*-GlcNAcylation and its upregulation during hyperglycemia is strongly correlated with the onset of insulin resistance and other diabetic complications (128) (131). The HBP will be discussed in more detail in section 1.7.

1.6.5 The Pentose Phosphate Pathway

1.6.5.1 Overview

Of the five NOGPs, only the PPP is proposed to play a beneficial role under hyperglycemic conditions (106). This pathway consists of both an oxidative and non -oxidative branch (132).

The first function of the PPP is to produce NADPH (oxidative branch) that plays an important antioxidant role and hence the reason this pathway is considered beneficial under hyperglycemic conditions (106). The second is to produce ribose-5-phosphate (non-oxidative branch) which is essential for nucleic acid biosynthesis and erythrose-4-phosphate (non-oxidative branch) for the biosynthesis of aromatic amino acids (133). Since the PPP is able to catalyze glycolytic intermediates such as fructose-6-phosphate back to pentose phosphatases, thereby reducing the concentrations of such intermediates and consequently diverting the flux away from the potentially harmful glycolytic pathways (134), it has generated many research investigations into its prospective beneficial role to treat type 2 diabetes (135). Increasing PPP flux may thus represent an opportunity to decrease ROS generation through the production of NADPH, and also potentially down - regulate flux through the above-mentioned damaging pathways (134). However, regulation of the PPP during times of hyperglycemia is controversial in the literature (138), i.e. some reported upregulated PPP with diabetes (138), while others demonstrated the opposite (139). Thus further studies are required to confirm its protective role under these circumstances.

1.6.5.2 Regulation

The rate-limiting enzyme of the non-oxidative branch of the PPP is transketolase and it catalyzes the reaction of pentose phosphatases to glycolytic intermediates and *vice versa* (132) (Figure 1.15). Since transketolase activity can be enhanced by benfotamine (a natural derivative of vitamin B1) altering the flux through the PPP has become a popular research topic in the treatment of type 2 diabetes (136) (137).

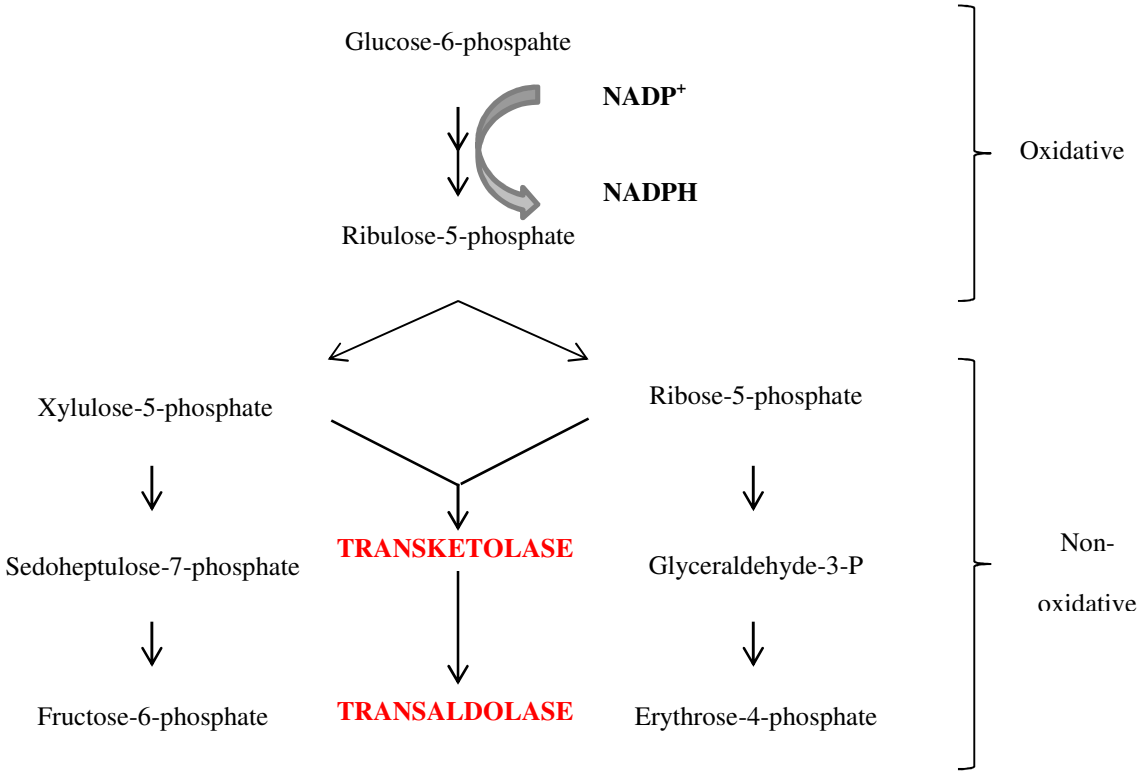

Figure 1. 15. Schematic representation of the PPP.

NADPH: Nicotinamide adenine dinucleotide phosphatase (reduced); $NADP^+$: Nicotinamide adenine dinucleotide phosphatase (oxidized).

Transketolase is encoded by the *TKT* gene that resides on chromosome Xq28 – a region associated with the formation of malignancies (140). In addition to transketolase, two transketolase-like proteins, transketolase-like 1 (TKTL1, encoded by *TKTL1*) and transketolase-like 2 (TKTL2, encoded by *TKTL2*) have been identified in the human genome and share a high sequence identity with TKT on both the gene and protein level (141,142). The primary sequence difference between TKT and TKTL1 is a deletion in the N-terminus (of 38 consecutive amino acids) of the latter, which forms part of the active site in transketolase (141). This has sparked several recent debates as to whether TKTL1 protein in fact does hold transketolase activity (143) as was previously hypothesized (141). *TKTL1* has recently been implicated in a wide variety of cancers – of which altered glucose metabolism is a hallmark – where its expression level has successfully been used as a marker for metastases and poor patient outcome (144) (145).

1.6.6 The activation of minor glycolytic pathways - recapped

The activation of alternative NOGPs is believed to be initiated by a single upstream metabolic event namely, mitochondrial superoxide overproduction (105) (106). Hyperglycemia results in augmented superoxide production which consequently induces DNA damage and in turn activates PARP (105). In addition to DNA repair, PARP inhibits the activity of the central glycolytic enzyme GAPDH (115), resulting in the accumulation of upstream glycolytic intermediates (106). Such intermediates are then shunted into five alternative glucose-utilizing pathways (106). Furthermore, flux through these pathways can also be augmented due to an increase in substrate (glucose) availability under hyperglycemic conditions, i.e. independent of GAPDH inhibition. Increased pathway flux subsequently results in ROS overproduction and a weakening in antioxidant capacity, leading to increased oxidative stress (107). The HBP has also been independently linked to the development of insulin resistance and type 2 diabetes (128). Despite the current lack of mechanistic detail linking the upregulation of alternative NOGPs to diabetes-associated pathologies, it is clear that these pathways are intertwined and that the activation of one detrimental pathway may stimulate the activation of others, resulting in a vicious feed-forward cycle of ROS production and accumulated cellular damage.

Since the HBP is not only upregulated with hyperglycemia, but has also been independently linked to the onset of insulin resistance and has the ability to execute cellular responses according to the nutritional status of the cell, it was the primary pathway under investigation in this study and will accordingly be discussed in full detail below.

1.7 THE HEXOSAMINE BIOSYNTHETIC PATHWAY: A REVIEW

The HBP is a fuel sensing pathway that modulates a significant number of cellular processes in response to the nutritional status of the cell, an action vital for cellular energy homeostasis (146). Such regulation is achieved through the PTM (by addition of *O*-GlcNAc molecules) of serine and/ or threonine residues of target proteins (147). Importantly, the HBP represents the amalgamation of glycolytic, amino acid, fatty acid, and nucleotide metabolism allowing for increased sensitivity towards the overall nutritional status of the cell (148) (149). Under physiological conditions ~2 - 5% of glucose is redirected from the primary glycolytic pathway into the HBP (128), where it is proposed to play a protective role in the cell against a wide variety of stress stimuli (150).

The HBP diverts from the main glycolytic pathway when fructose-6-phosphate is converted to glucosamine-6-phosphate by the rate-limiting enzyme GFAT (129). Glucosamine-6-phosphate is then converted – via a series of reactions – to UDP-GlcNAc, the HBP end product (129). UDP-GlcNAc subsequently serves as a substrate for the dynamic attachment of *O*-GlcNAc moieties to the hydroxyl groups of serine and/ or threonine residues of target proteins – a process commonly known as *O*-GlcNAcylation (147) (Figure 1.16). The *O*-GlcNAc modification is dynamic, reversible and tightly regulated by only two known enzymes, *O*-linked β-N-acetylglucosamine (GlcNAc) transferase (*O*-GlcNAc transferase, OGT) and β-N-acetylglucosaminidase (*O*-GlcNAcase, OGA) that catalyze the addition and removal of *O*-GlcNAc moieties, respectively (Figure 1.16). Glucosamine is better able to increase levels of UDP-GlcNAc since it enters the HBP downstream of the rate-limiting enzyme GFAT (128) (151) (Figure 1.16). This dynamic and reversible modification is fast emerging as a key regulator of a wide variety of cellular processes; consequently

perturbations in *O*-GlcNAcylation have been associated with a number of pathophysiological conditions including neurodegenerative diseases, cancer and type 2 diabetes (152).

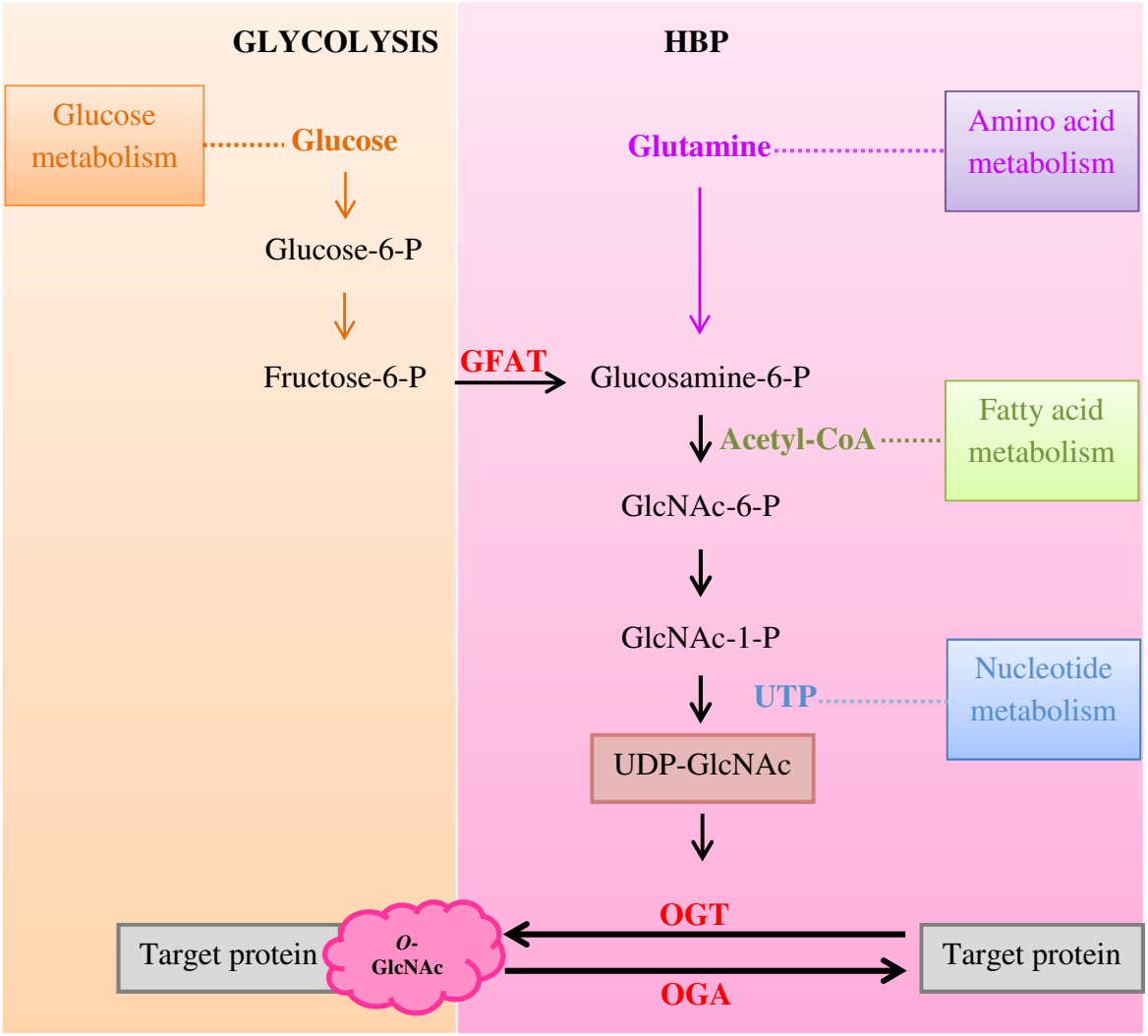

Figure 1. 16. The HBP represents an amalgamation of different metabolic inputs.

The HBP (pink) diverts from glycolysis (orange) when fructose-6-phosphate is converted to glucosamine-6-phosphate by GFAT, the rate-limiting enzyme. The HBP culminates in the attachment/ removal of *O*-GlcNAc moieties onto target proteins by OGT and OGA, respectively. The HBP has the ability to integrate additional metabolic inputs including amino acid metabolism (purple), fatty acid metabolism (green), and nucleotide metabolism (blue).

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.

1.7.1 Experimental evidence links the HBP to insulin resistance and type 2 diabetes

1.7.1.1 Insulin resistance

The association between the HBP and insulin resistance was first described by Marshall et al. in 1991. Here a marked decrease in insulin sensitivity was observed in isolated rat adipocytes when cells were exposed to high glucose concentrations and glucosamine, respectively, in the presence of insulin. This effect was reversed by reducing HBP flux – by using GFAT inhibitors (128). Numerous studies (cell culture and animal models) link the HBP to the development of insulin resistance. For example, transgenic mice overexpressing GFAT in both skeletal muscle and adipose tissue develop insulin resistance (153-155). Moreover, elevation of intracellular *O*-GlcNAc levels by either OGT over-expression or OGA inhibition also resulted in the manifestation of insulin resistance (153) (156-158).

1.7.1.2 Type 2 diabetes

A small subset of studies have linked alterations in the HBP with clinical type 2 diabetes. Here two studies demonstrated *O*-GlcNAcylation of target proteins occurs in a glucose-responsive manner and that there were significant differences between control, pre – diabetic and diabetic individuals for global *O*-GlcNAcylation and OGA protein expression (43) (159). A mutation in the *OGA* gene has also been implicated in the development of type 2 diabetes in the Mexican American population, further implicating altered HBP flux in the development of type 2 diabetes (67). Thus there is strong support for the link between increased HBP flux and the development of insulin resistance, and the underlying mechanism responsible for this causal link appears to be aberrant phosphorylation (160).

1.7.1.3 Mechanism

O-GlcNAcylation is a dynamic and reversible PTM and exhibits complex and extensive cross-talk with phosphorylation since they regularly compete for many of the same binding sites on target proteins (160). For example, in a study where over 700 phosphorylation sites were monitored after a 3-fold increase in global *O*-GlcNAcylation, the phosphorylation activity at almost every site was significantly altered in response to *O*-GlcNAc modification (161). Furthermore, *O*-GlcNAcylated or phosphorylated residues can be in nearby proximity to one another and sterically hinder the attachment of the other modification (160). *O*-GlcNAcylation possesses the ability to rival phosphorylation and disrupt/ interfere with vital phosphorylation signaling cascades – a concept known as the "Yin-Yang hypothesis" (162). Therefore during times of hyper *O*-GlcNAcylation the attachment of *O*-GlcNAc moieties to target proteins disrupts essential phosphorylation cascades, interfering with many vital signaling pathways (158). Moreover, the *O*-GlcNAc status of a protein impacts on protein folding, catalytic activity and cellular localization, ultimately affecting its downstream function(s) (Reviewed in (163)). Hyper *O*-GlcNAcylation has therefore been identified as the underlying phenotype behind various disease etiologies including; neurodegenerative diseases, cancer, and type 2 diabetes (recently reviewed in (146)).

Since insulin signaling is dependent on phosphorylation, it is an example of a metabolic pathway exceedingly susceptible to disruption by *O*-GlcNAcylation (158). In support, studies found that increased *O*-GlcNAcylation inhibits insulin signaling by preventing IRS1 and AKT phosphorylation by *O*-GlcNAc modification (164). More recently it was found that after insulin stimulation, PIP3 recruited OGT from the nucleus to the sarcolemma where *O*-GlcNAcylation, rather than phosphorylation, of key insulin signaling molecules was favored thereby diminishing insulin signaling (158). This ultimately impacts negatively upon glucose

homeostasis (discussed in detail in sections *1.4.2.1 - 1.4.2.4*). *O*-GlcNAcylation of β-cell and mitochondrial proteins can also disturb insulin secretion and promote mitochondrial dysfunction, respectively, further exacerbating insulin resistance and contributing to glucose toxicity (165) (166). These effects also extend to the transcriptional level, e.g. FOXO is susceptible to *O*-GlcNAc modification thereby resulting in increased expression of gluconeogenic genes together with greater hepatic glucose output (167). The profound effect *O*-GlcNAc has on insulin signaling is only beginning to unravel (146).

While the similarity between *O*-GlcNAcylation and phosphorylation is striking it must be noted that while phosphorylation is controlled by hundreds of kinases and phosphatases, *O*-GlcNAc cycling is under the control of only two enzymes (168).

1.7.3 HBP Regulation

O-GlcNAcylation is under the stringent control of two evolutionary conserved enzymes, OGT and OGA (168). OGT is responsible for the attachment of a single *O*-GlcNAc molecule to the hydroxyl groups of serine/ threonine residues on target proteins while OGA catalyzes the removal of *O*-GlcNAc moieties from modified proteins (168).

1.7.3.1 OGT

OGT catalyzes the attachment of *O*-GlcNAc moieties to over 1000 nuclear and cytoplasmic target proteins (146). These include proteins involved in cell signaling, transcription, translation, the cell cycle and many more (160) (169). OGT is found in nearly all living organisms from plants to higher mammals (170), is expressed in all tissue types examined thus far, and exhibits high species homology. The human *OGT* gene resides on the X

chromosome at position Xq13.1 (171) and the deletion or disruption thereof has proven lethal (as demonstrated by OGT knock-out studies (172)). Although OGT is encoded by a single highly conserved gene in mammals, alternative splicing results in three distinct isoforms: nuclear-cytoplasmic OGT (ncOGT); mitochondrial OGT (mOGT) and short OGT (sOGT) (171) (173) (174). The three isoforms share an identical C-terminal domain that confers catalytic activity, but differ in their N-terminal domain regarding the number of tetratricopeptide (TPR) domains (168). The TPR domains consist of 34 highly conserved consecutive amino acids that are repeated between 3 - 12 times and mediate OGT substrate specificity and localization to different subcellular compartments (160) (175). The expression of each isoform differs among various tissues and cell types, suggesting that different isoforms may have distinct functions (176). Since a single enzyme is exclusively responsible for the attachment of *O*-GlcNAc moieties to thousands of target proteins with no obvious consensus sequence, *OGT* regulation remains intriguing and is yet to be fully elucidated (177). However, OGT is also regulated in a number of other ways including: its binding to lipids; phosphorylation; and auto-*O*-GlcNAcylation, albeit their mechanisms remain vague (163). As the tools used for the detection of *O*-GlcNAc become more sensitive and more *O*-GlcNAc modified proteins are identified (146), it becomes clear that the regulation of OGT activity and substrate specificity is highly complex and still poorly understood (163).

1.7.3.2 OGA

OGA is derived from a single highly conserved gene mapping to position 10q24.1 - q24.3. OGA and meningioma expressed antigen 5 (MGEA5) were shown to be encoded by the same gene (178). *OGA* has at least two know alternative spliced forms, one of > 100 kDa and the other shorter ~70 kDa isoform (168). Little is known about the different functions or localization of these two *OGA* isoforms, although the larger isoform appears to display

greater catalytic activity (179). Since OGA is able to bind thousands of diverse proteins its regulation – as with OGT – has attracted interest (168).

1.7.3.3 GFPT1 and GFPT2

In addition to the direct regulatory role that OGT and OGA play in *O*-GlcNAc cycling, GFAT is responsible for flux through the HBP, ultimately affecting UDP-GlcNAc concentrations – the substrate for *O*-GlcNAcylation (151). GFAT is considered the rate-limiting enzyme of the HBP owing to feedback inhibition by the product itself (glucosamine-6-phosphate) and the end product UDP-GlcNAc (151). GFAT is highly conserved among species and is regulated on both a post-transcriptional and post-translational level (148) (180). The enzyme is transcribed by two distinct genes, *GFPT1* (chromosome 2p13) (181) and *GFPT2* (chromosome 5q34 - q35) (182) with differing tissue distributions, raising the possibility that they may have distinct roles in the development of diabetic complications (183). Indeed, both genes and their respective enzymes were previously implicated in the pathogenesis of type 2 diabetes. Here diabetic patients exhibited increased *GFPT2* mRNA levels in lymphocytes (184), while they found augmented GFAT activity in skeletal muscle (185). (also refer back to section *1.7.1.1* for additional cell culture and animal model studies).

Despite *O*-GlcNAcylation being a ubiquitous PTM with far reaching biological implications, it is essentially controlled by only three regulatory enzymes (GFAT, OGT and OGA) encoded for by four genes (*GFPT1; GFPT2; OGT* and *OGA*). This singularity in combination with the global disposition of the *O*-GlcNAc modification highlights the critical and fundamental nature of *O*-GlcNAcylation.

1.8 SUMMARY

Type 2 diabetes has reached epidemic proportions and its association with diseases such as CVD results in increased morbidity and mortality rates (16). Alarmingly, diabetes prevalence is estimated to double over the next 20 years (7).

Despite the availability and accessibility of current diagnostic tools, diabetes remains relatively poorly diagnosed, especially in poorer countries (22). Moreover, the number of patients presenting with diabetes-associated complications at diagnosis is unacceptable (23). The controversies surrounding specific diagnostic thresholds in combination with the shortcomings of each assay highlights the need for investigation into novel diagnostic concepts to assist with the earlier and more efficient detection of type 2 diabetes.

Hyperglycemia, the biochemical hallmark of diabetes, promotes the formation of ROS via the uncoupling of the mitochondrial ETC (106). Consequently flux through the five non-oxidative minor branches of glycolysis is elevated resulting in diabetes-associated micro- and macrovasculature pathologies (106). This sets the stage for our hypothesis:

1.9 HYPOTHESIS

Since flux through the NOGPs is increased in response to hyperglycemia, we hypothesize that genes encoding the rate-limiting enzymes of each respective pathway will be differentially expressed between control, pre-diabetic and diabetic individuals. Moreover, differential gene expression in response to augmented glucose flux may represent an exploratory avenue in the search for a novel diagnostic tool(s) for type 2 diabetes.

1.10 AIMS AND OBJECTIVES

- Recruit volunteers and acquire blood specimens representing a variety of fasting blood glucose levels, from normal to pre-diabetic and diabetic.
- Characterize individuals according to ADA and WHO criteria based on fasting plasma glucose and HbA1c levels, respectively.
- Design and optimize seven primer pairs for the genes under investigation.
- Extract mRNA from volunteers and measure leukocyte mRNA transcript levels of: *GFPT1; GFPT2; OGT; OGA; TKT; TKTL* and *AKR1B1* employing quantitative real-time PCR (qPCR).
- Determine differences in mRNA transcript levels between control, pre-diabetic and diabetic individuals.

In doing so we hope to make a significant scientific contribution to the advancing field of *O*-GlcNAcylation and its role as a candidate in the search for a novel diagnostic tool for type 2 diabetes. Furthermore, we hope to identify additional biomarkers that are differentially expressed with the onset of type 2 diabetes mellitus.

Chapter 2

EXPERIMENTAL PROCEDURES

2. EXPERIMENTAL PROCEDURES

2.1 SUBJECT RECRUITMENT AND CHARACTERIZATION

This study was accepted by the Committee for Human Research at Stellenbosch University (reference number: S12/03/074) and was carried out according to the ethical guidelines and principles of the International Declaration of Helsinki, the Medical Research Council Ethical Guidelines for Research in South Africa, and the South African Guidelines for Good Clinical Practice. Experimental design and execution was carried out according to the Minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (186). Volunteers (n = 60; n=20 Mixed Ancestry, n=40 Caucasian) were recruited from two neighboring metropolitan regions, namely Stellenbosch and Paarl (Western Cape, South Africa). All recruited participants were personally informed about the study and were requested to sign a written consent form detailing the study aims and procedures.

Due to the aforementioned discrepancies surrounding the diagnosis of type 2 diabetes, we grouped participants according to their FPG levels (ADA and WHO criteria) and their HbA1c levels, respectively. Since the WHO does not endorse the use of HbA1c to differentiate between pre-diabetes and diabetes, patients classified according to HbA1c levels were done so using the ADA criteria. Thus each participant was assigned to one of three groups: control; pre-diabetic; or diabetic according to three different diagnostic criteria (refer to Table 2.1). Clinical information regarding our study participants is summarized in Table 2.2.

Table 2. 1. Subject characterization

1. FPG ADA Criteria	2. FPG WHO Criteria	3. HbA1c ADA Criteria
	Control	
<5.6 mmol/l	<6.1 mmol/l	<5.5%
	Pre-diabetic	
5.6 - 6.9 mmol/l	6.1 - 6.9 mmol/l	5.5 - 6.5%
	Diabetic	
>7 mmol/l	>7 mmol/l	>6.5 %

ADA, American Diabetes Association; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin.

Table 2. 2. Summary of subjects' details

	Controls	Pre-diabetic	Diabetic
Sample size	30	14	16
Age (years)*	50 ± 2.3	55.4 ± 4	54.8 ± 2.4
Gender M/F	6/24	7/7	6/10
Ethnicity MA/C	7/23	6/8	7/9
FPG (mmol/l)*	5 ± 0.07	6.3 ± 0.1	10 ± 0.8
HbA1c (%)*	5.6 ± 0.08	6.6 ± 0.2	8.7 ± 0.5
Insulin (mIU/l)*	9.1 ± 1.1	14.3 ± 3.6	32.3 ± 11.5

C, Caucasian; F, Female; FPG, fasting plasma glucose; M, Male; MA, Mixed Ancestry

* Values expressed as mean ± SEM

2.2 SPECIMEN COLLECTION

Whole blood samples were collected from participants, under fasting conditions, by venipuncture into specified tubes provided by PathCare, Stellenbosch (Western Cape, South Africa). Clinical measurements included: fasting blood glucose (4 ml potassium oxalate/

sodium fluoride tube), HbA1c (4 ml EDTA tube), insulin (5 ml serum separating tube), C-reactive protein (5 ml serum separating tube) and homocystein (4 ml EDTA tube). For molecular studies, collected blood samples (4 ml EDTA tube) were stored on ice while transported, and total RNA extracted within 3 hours of sample collection.

2.3 RNA EXTRACTION & PRECIPITATION

Total RNA was extracted using the QIAamp RNA Blood Mini kit in combination with the RNase-Free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To confirm the quality and quantity of extracted RNA, samples were subjected to agarose gel electrophoresis and spectrophotometry, respectively. A 2% (w/v) RNase-free agarose gel was prepared using 1X TBE buffer (1m M EDTA, 90 mM Tris, 90 mM boric acid dissolved in 1L RNase-free water) and 0.01% (v/v) ethidium bromide (EtBr). The gel was loaded with 2.5 µl of sample RNA, stained with 1X RNA loading dye (Fermentas, ThermoFisher Scientific, Waltham, MA, USA) and 5 µl of High Range RiboRuler RNA ladder (Fermentas, ThermoFisher Scientific, Waltham, MA, USA) respectively, after being heated at 70ºC for 5 min in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). Electrophoresis proceeded for 1 hour at 120 V in 1X TBE buffer where after the image was visualized using ultra violet light on a Multigenius BioImaging System (Syngene, Cambridge, UK). Additionally, each RNA sample was subjected to spectrophotometry (NanoDrop® ND-1000 spectrophotometer V3.0.1, NanoDrop Technologies, Wilmington, DE, USA) in order to determine the respective concentrations and A260/A280 purity ratios. Samples with an A260/A280 ratio of <1.8 were discarded. Samples were stored at -80ºC (in 3 volumes of 100% ethanol) until all 60 samples had been collected.

RNA precipitation was performed using a standard sodium acetate protocol. Samples were removed from the -80ºC freezer, allowed to thaw on ice, whereafter sodium acetate was directly added to achieve a final concentration of 0.3 M. Afterwards, samples were immediately placed at -80ºC for 20 min and subsequently centrifuged at 12, 000 × *g* at 4ºC for 15 min, and the supernatant thereafter discarded. Next, 500 µl of 75% ethanol was added and samples were again centrifuged at 12, 000 × *g* at 4ºC for 15 min. After discarding the supernatant, RNA pellets were air-dried and dissolved in RNase-free water (Qiagen, Hilden, Germany) to a final concentration of 200 ng/µl.

2.4 cDNA SYNTHESIS

First strand cDNA synthesis was performed using the Go Script™ Reverse Transcription System (Promega, Fitchburg WI) according to the manufacturer's guidelines. A total of 2.5 µl of RNA (200 ng/µl) was transcribed to 20 µl of subsequent cDNA. In addition to study samples a no template control and a no reverse transcriptase control were included to monitor contamination. Transcribed cDNA was diluted 10-fold in RNase-free PCR grade water (Roche Diagnostics, Mannheim, Germany) in order to create a surplus of sample cDNA. cDNA samples were stored at -20°C and expression levels measured within 1 week. All target gene were run using 10-fold diluted cDNA, except *GFPT1* and *GFPT2* which were run using concentrated cDNA (prior to performing the 10-fold dilution).

2.5 GENE EXPRESSION ANALYSIS

2.5.1 Quantitative real-time PCR

2.5.1.1 Oligonucleotide Primer design

Although various detection chemistries are available for gene expression studies, SYBR Green I dye was chosen for its sensitivity and reduced running costs (187). Despite these advantages an impediment of SYBR Green 1 technology is its ability to bind to all double-stranded DNA in a non-specific manner (187) (Figure 2.1A). This increases the chance of false positives since the dye will bind to specific and non-specific double stranded PCR products. Due to this phenomenon great precautions had to be taken when designing primers. This was important to ensure that the fluorescent signal being detected was indeed from the target gene and not from non-specific double stranded PCR products, contaminating genomic DNA (gDNA), or primer dimers. This allowed for the accurate measurement of target gene expression.

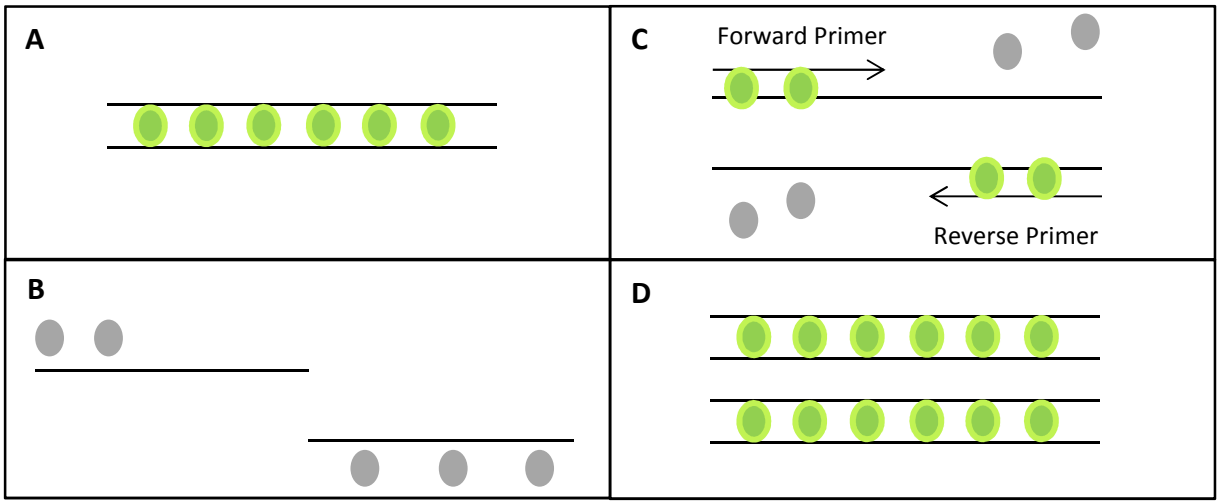

Figure 2. 1. A graphic representation of SYBR Green I detection chemistry.

A. Reaction set up: SYBR Green I dye binds to all dsDNA non-specifically and consequently fluoresces. **B. Pre-incubation/ elongation:** an increase in temperature causes dsDNA to denature and SYBR Green I to be released resulting in a decreased fluorescent signal. **C. Elongation:** Primers anneal and dsPCR product is formed causing SYBR Green I to bind. **D. End of elongation:** SYBR Green I is bound to all amplified dsPCR product with maximum fluorescence proportional to the amount of PCR product produced. A fluorescence reading is obtained at this point.

Reference sequences for all genes investigated herein were obtained from the Ensembl database (www.ensembl.org). Subsequently, primer pairs were designed using PrimerQuest software (Intergrated DNA Technologies) which is an open resource and can be accessed at https://eu.idtdna.com/PrimerQuest/Home/Index. Since contaminating gDNA remains an undesired reality in qPCR, primers were designed to anneal to two consecutive exons with an intron spanning >1 kb between the exons. Consequently, only cDNA from mRNA transcripts would be amplified and not transcripts from larger contaminating gDNA, allowing for an accurate reading of target gene expression levels. In order to eliminate the presence of primer dimers, primer self-complementarity was assessed. Finally, all sequences were entered into NCBI BLAST in order to evaluate whether the designed primer sequences would indeed bind to the gene of interest, and consequently amplify the target gene, and to check if the primer sequence shared homology with any non-specific targets which would cause the amplification of undesired PCR products. Once all the above specifications were met for each primer pair, primers were ordered from Inqaba Biotech (Pretoria, South Africa). Primer pairs for Beta actin *(ACTB),* and Ribosomal protein 37A (*RPL37A*) had been previously designed (188). Refer to Table 2.3 for primer sequences.

Table 2. 3. Primer sequences and optimal PCR conditions for the amplification of synthesized cDNA

HUGO nomenclature	Ensembl Accession Number	Primer	Primer Sequence (5'- 3')	Cycle Number	Product Size (bp)	T_M (°C)	T_A (°C)
ACTB[*]	ENSG00000075624	F	ATTGCCGACAGGATGCAGAA	40	150	57.1	60
		R	GCTGATCCACATCTGCTGGAA			57.4	
AKR1B1	ENSG00000085662	F	CTACCTTATTCACTGGCCGACT	40	169	56.3	60
		R	GTTGGAGATGCCAATAGCTTTC			54.1	
GFPT1	ENSG00000198380	F	TGCTGTGCTGAGAGGCTATGATGT	40	358	60.2	65
		R	GTCCAGAAATGCAACACCCAGCAT			60.2	
GFPT2	ENSG00000131459	F	GGGCGACAAGGCCGTGGAAT	40	107	63.1	60
		R	CAGCCACTGCGGCGATGTCA			63.1	
GUS	ENSG00000169919	F	CTCATTTGGAATTTTGCCGATT	40	81	57.3	55
		R	CCGAGTGAAGATCCCCTTTTTA			59.9	
OGA	ENSG00000198408	F	GCAGCACCCTCTTTAAATGCCACA	40	358	60.2	60
		R	CCTGGCACAAACTGCTCCTTGTTT			60.3	
OGT	ENSG00000147162	F	GCAACGTGGCCGACAGCACA	40	117	63.7	62
		R	TGCAGTGTCTCTCAGCTGCCTCA			62.4	
RPL37A1[*]	ENSG00000145592	F	CGCCCAGAAGGTGACCAATGC	40	203	61.2	60
		R	ATGCAACTGGAGGGAACTGGG			61.0	
TKT	ENSG00000163931	F	TACCGAGTCTATTGCTTGCTGGGA	40	289	59.7	60
		R	AGGTCTTGGCAATGATGGCTGTTG			60.0	
TKTL1	ENSG00000007350	F	TTGTCCTCGCAAAGAGACTGTCGT	50	296	60.2	65
		R	TTCGCAGCGCCTCTGATAGATGTT			60.5	

[*]Primers previously designed (188).

5': 5-prime; 3': 3-prime; °C: degrees Celsius; *ACTB*: β-actin; *AKR1B1:* Aldo-keto reductase family 1, member B1; bp: base pairs; F: Forward; *GFPT1*: glutamine:fructose-6-phosphate amidotransferase 1; *GFPT2*: glutamine:fructose-6-phosphate amidotransferase 2; *GUS*: β-glucuronidase; *OGA*: O-GlcNAcase; *OGT*: O-GlcNAc transferse; R: Reverse; *RPL37A*: ribosomal protein L37a; TKT: Transketolase; TKTL1: Transketolase-like protein 1; T_A: Annealing temperature; T_M: Melting temperature.

2.5.1.2 Optimization and standard curve generation

Random cDNA samples were used to establish the optimal primer conditions (refer to Table 2.3). Annealing temperature, cycle number and primer concentration were manipulated in order to optimize target gene amplification. To confirm that the PCR conditions were indeed optimal a melt-curve analysis was performed to ensure that one discreet peak was detected, since this indicated that only one product was being amplified. To confirm that this product was the target gene, and not additional non-specific product/ primer dimer, samples were subjected to agarose gel electrophoresis followed by UV imaging allowing for confirmation of the expected product size.

Standard curves were generated for each target and reference gene examined. For this, random control samples were amplified, pooled and purified using the GenElute PCR clean up kit (Sigma Aldrich, St. Louis, MO, USA) according to the supplied protocol. Subsequently, the concentration of the pooled product was measured using spectrophotometry (NanoDrop® ND-1000 spectrophotometer V3.0.1, NanoDrop Technologies, Wilmington, DE, USA). To generate standard curves the diluted product was further serially diluted in RNase-free PCR grade water over a concentration range of 10^{0} - 10^{-5}. To produce a reliable standard curve for each gene, the product was amplified in replicates of 5 for each dilution point over the entire concentration range. The serial dilutions used to generate the standard curves were stored at -20ºC and used for validation of imported standard curves. Efficiency and slope values for the standard curves generated are presented in Table 2.4.

Table 2. 4. Standard curve statistics

Gene	Efficiency	Slope
ACTB	1.99	-3.34
AKR1B1	1.96	-3.43
GFPT1	1.95	-3.44
GFPT2	1.97	-3.40
GUS	2.00	-3.20
OGA	1.91	-3.50
OGT	1.99	-3.32
RPL37A	1.98	-3.37
TKT	1.98	-3.36
TKTL1	1.98	-3.37

ACTB: β-actin; *AKR1B1:* Aldo-keto reductase family 1, member B1; *GFPT1*: glutamine:fructose-6-phosphate amidotransferase 1; *GFPT2*: glutamine:fructose-6-phosphate amidotransferase 2; *GUS*: β-glucuronidase; *OGA*: O-GlcNAcase; *OGT*: O-GlcNAc transferse; R: Reverse; *RPL37A*: ribosomal protein L37a; TKT: Transketolase; TKTL1: Transketolase-like protein 1.

2.5.1.3 Amplification of cDNA Sequences

Target gene amplification was carried out on the Roche LightCycler 480 system in 10 µl reactions. Each reaction contained 2 µl of 2x Lightcycler® 480 SYBR Green I Master, 0.5 pmol of each respective forward and reverse primer, and 1 µl of the corresponding cDNA sample. Samples were run in triplicate on two separate occasions each time employing a negative control, no template control, no reverse transcriptase control and the respective standard curve serial dilution to validate the PCR efficiency and calculate the relative sample concentrations. A calibrator was also employed in order to eliminate inter-run variability (Refer to Figure 2.2 for an example of plate setup). The reactions were cycled with a pre-incubation step for 10 min at 95ºC; amplification for 15 sec at the annealing temperature (T_A) (see Table 2 for optimal T_A and cycle number); elongation for 20 sec at 72ºC. Fluorescence was measured at the end of the elongation phase at 72ºC.

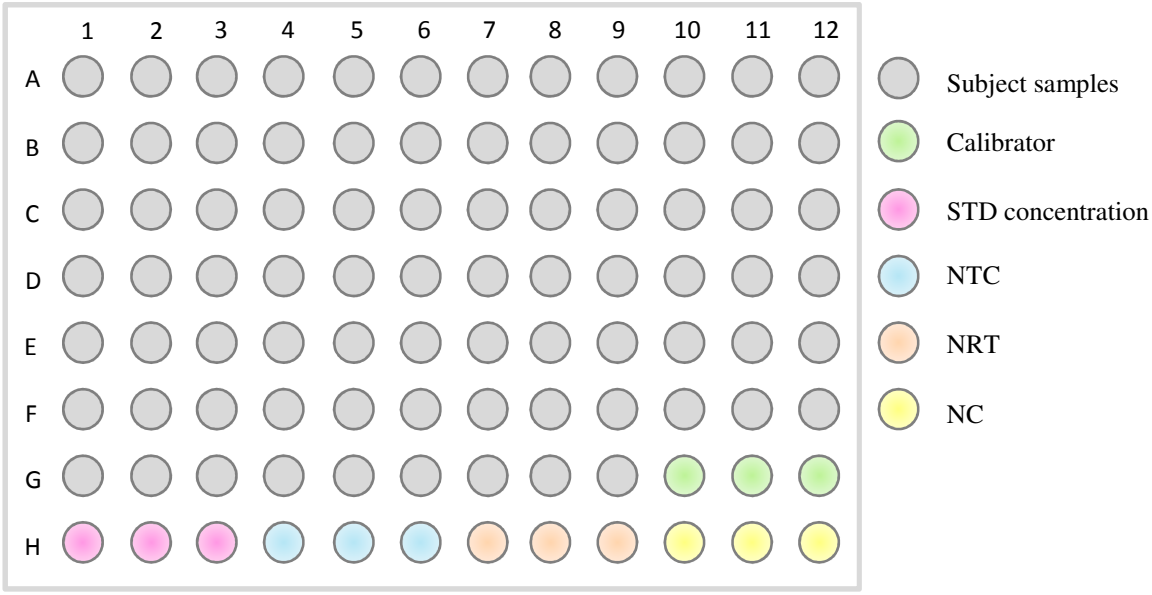

Figure 2. 2. Graphical representation of a 96-well LightCycler plate setup.

NC, negative control; NRT, no reverse transcriptase control; NTC, no template control; STD, standard.

2.6 DATA AND STATISTICAL ANALYSIS

Standard curves were imported into subject recruit sample runs and used to calculate the relative concentrations for each target gene, respectively. Relative concentration values were averaged per patient sample and normalized to a calibrator. The relative levels of target gene expression were normalized against *GUS*, *ACTB* and *RPL37A* expression, respectively. This was carried out in two independent experimental runs and the values generated from rounds 1 and 2 were averaged and used for downstream statistical analyses. One-way analysis of variance (ANOVA) was used to determine differences in gene expression ratios between normal, pre-diabetic and diabetic individuals, followed by two Bonferroni *post hoc* tests. One that compared all pairs of columns and one that compared selected pairs of columns (denoted with ⊢—⊣). All statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software Inc, San Diego, CA, USA). P values < 0.05 were recognized as significant. All values are expressed as the mean ± SEM.

Chapter 3

RESULTS

3. RESULTS

3.1 INVESTIGATION INTO THE HBP

Here we measured *OGA, OGT, GFPT1* and *GFPT2* mRNA transcript levels using qPCR with SYBR Green 1 detection chemistry. Genes under investigation were normalized to three individual reference genes (*GUS, ACTB* and *RPL37A*) in order to validate findings. A conclusion was drawn if two out of three reference genes presented similar results. Study participants were characterized according to: 1) FPG levels, ADA criteria; 2) FPG levels, WHO criteria and 3) HbA1c levels, ADA criteria.

3.1.1 Attenuated *OGA* expression is associated with diabetes

3.1.1.1 Reduced OGA expression with increased fasting blood glucose levels (ADA)

We firstly investigated the mRNA transcript levels of *OGA* in pre-diabetic and diabetic individuals that were characterized according to FPG levels (ADA criteria). By employing the use of three reference genes we concluded that decreased *OGA* expression levels accompany diabetes. Both *GUS* and *ACTB* normalized data demonstrated a decrease in *OGA* expression levels between control and diabetic individuals of 33 ± 7.8% and 31.7 ± 7.5%, respectively (Figure 3.1 A, B). Additionally, *OGA* normalized to *GUS* was able to distinguish between pre-diabetic and diabetic volunteers with a decrease in *OGA* expression of 24.3 ± 8.6% in the diabetic group compared to the pre-diabetics (Figure 3.1 A). While *OGA* normalized to *ACTB* could differentiate between control and pre-diabetic participants, demonstrating a 22.5 ± 9.7% reduction (Figure 3.1 B). *RPL37A* normalized data did not display any statistically significant changes (Figure 3.1 C).

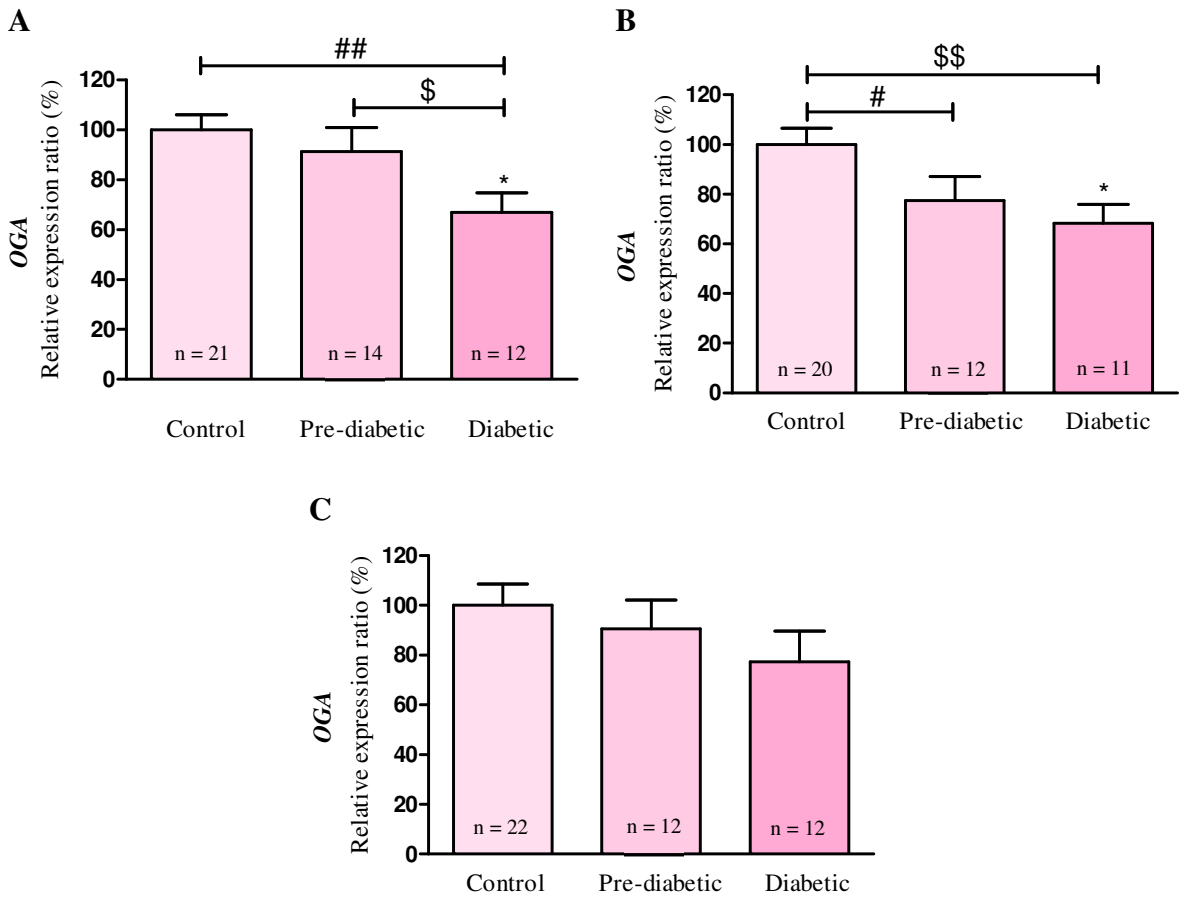

Figure 3. 1. Decreased *OGA* expression in ADA characterized diabetic individuals (*GUS, ACTB* and *RPL37A* normalized).

A: Decreased OGA/GUS expression levels in diabetic individuals compared to controls (n=47; *p<0.05; ##p<0.01) and pre-diabetic participants (n=47; $p<0.05). B: Attenuated OGA/ACTB expression in both diabetic (n=43; *p<0.05; $$p<0.01) and pre-diabetic (n=43; #p<0.05) groups versus control individuals. C. No significant differences were identified in OGA/RPL37A expression levels between the three study groups (n=46).

3.1.1.2 OGA expression is able to detect the onset of pre-diabetes (fasting blood glucose, WHO criteria)

When study participants were re-characterized according to the WHO FPG criteria, a decrease in *OGA* expression levels of (34.9 ± 8.2%; 33.9 ± 8%; and 33.4 ± 11%) between diabetic and control individuals was observed with normalization to all three reference genes: *GUS; ACTB* and *RPL37A*, respectively (Figure 3.2 A, B, C). Interestingly, *OGA* expression was also able to detect the difference between control and pre-diabetic individuals (23.7 ±

9.6% and 32 ± 9.8 % reduction) when normalized to *GUS* and *ACTB*, respectively (Figure 3.2 A, B).

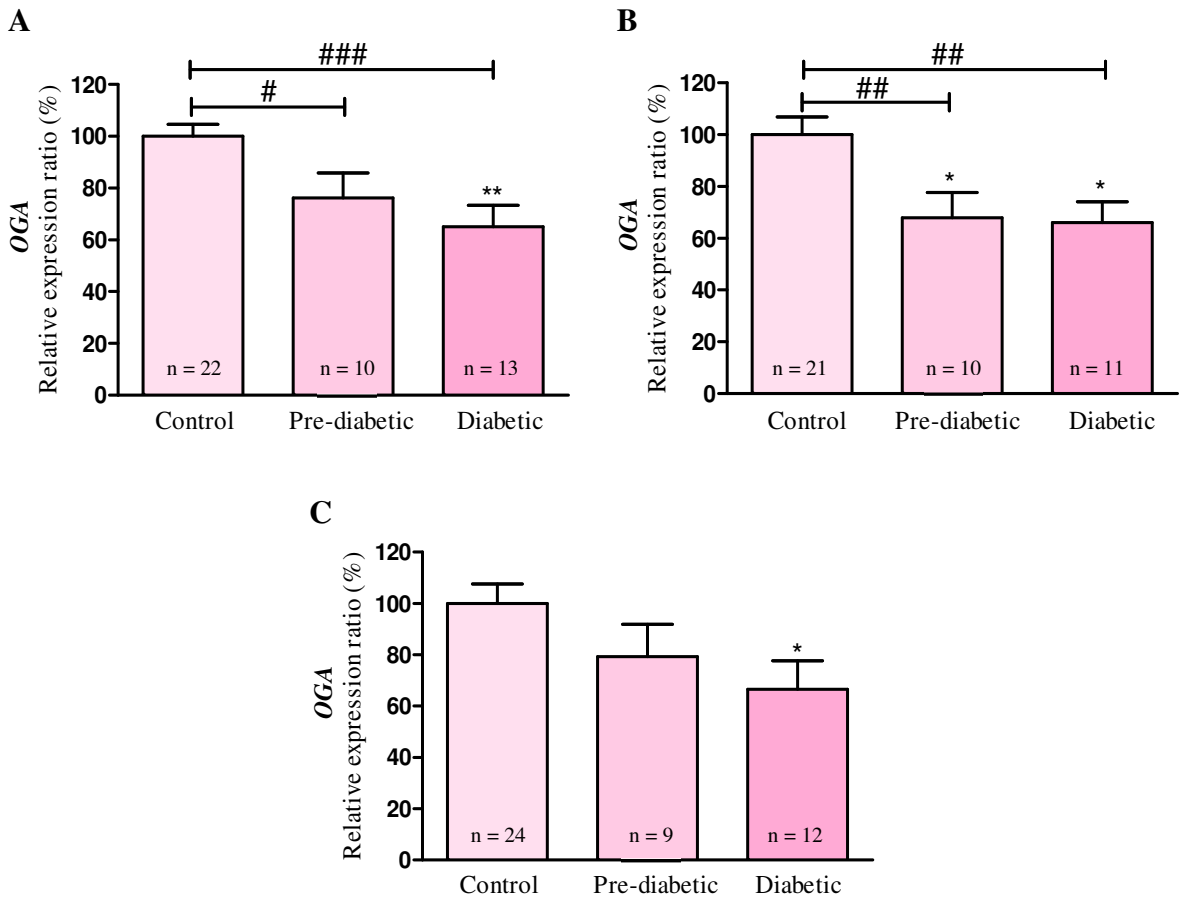

Figure 3. 2. *OGA* expression is attenuated with increasing FPG concentrations at the pre-diabetic and diabetic level (WHO criteria).

A: Lower *OGA/GUS* in diabetic (n=45; **p<0.01; ###p<0.001) and pre-diabetic (n=45; #p<0.05) individuals compared to controls. **B:** *OGA/ACTB* shows decreased levels in diabetic (n=42; *p<0.5; ##p<0.01) and pre-diabetic (n=42; *p<0.05; ##p<0.01) groups when compared to control individuals. **C:** *OGA/RPL37A* is decreased in diabetics *vs.* controls (n=45; *p<0.05).

3.1.1.3 OGA expression decreases with increasing HbA1c levels (ADA)

A similar trend was observed in *OGA* expression when patients were grouped according to HbA1c levels (ADA criteria). All three reference genes indicated a decrease in *OGA* expression levels of 35.7 ± 6.3% (*GUS*), 33.5 ± 6.6% (*ACTB*), and 23.3 ± 8.7% (*RPL37A*) in

diabetic volunteers compared to controls (Figure 3.3 A, B, C). Moreover, contrary to previous classifications, *OGA* normalized to *GUS* and *ACTB* were able to distinguish the pre-diabetic from the diabetic group showing a decrease of 29.1 ± 6.3% and 32.3 ± 6.6%, respectively (Figure 3.3 A, B).

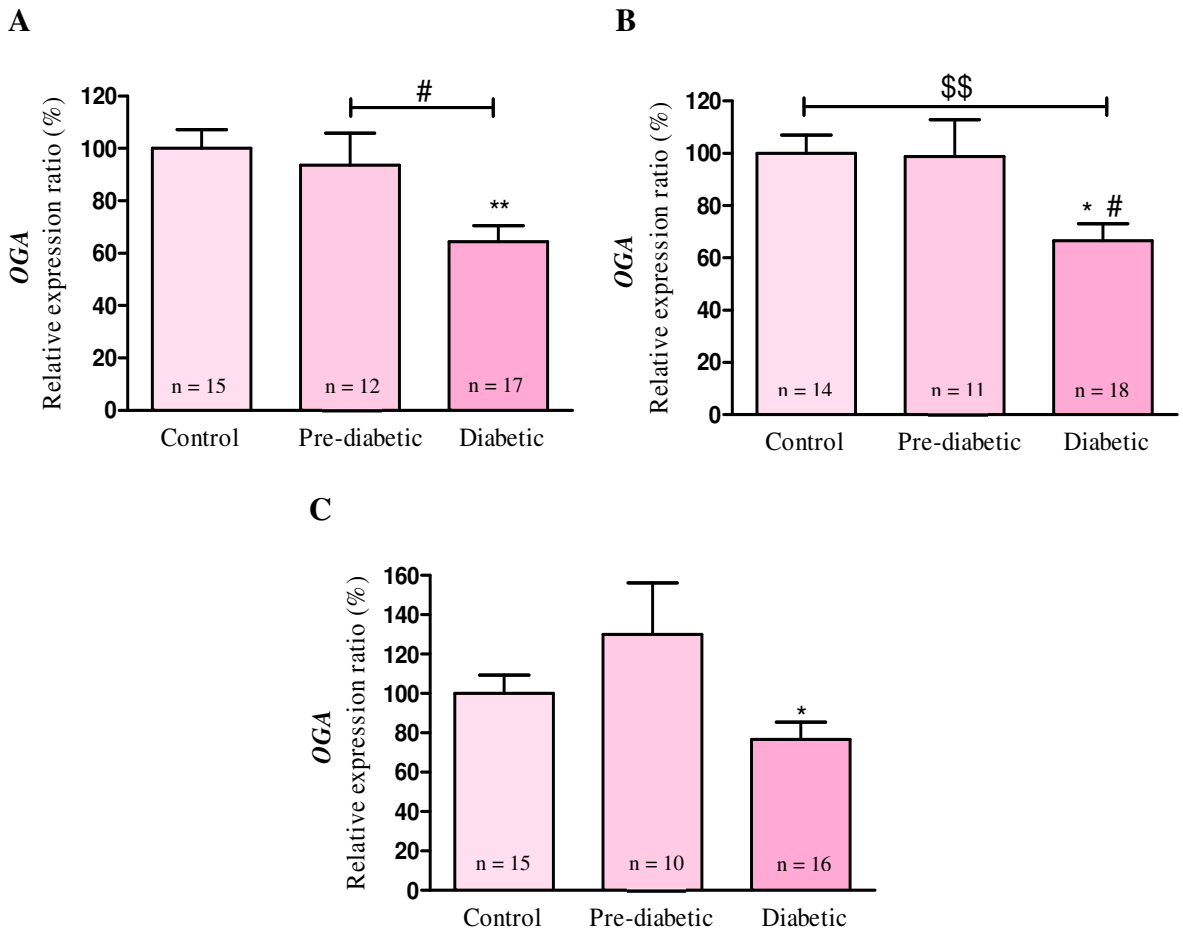

Figure 3. 3. *OGA* expression levels decrease with increasing HbA1c levels (ADA criteria).

A: Decreased *OGA/GUS* expression levels in diabetic *vs.* control groups (n=44; **p<0.01) pre-diabetic groups (n=44; #p<0.05). **B:** *OGA/ACTB* expression levels are reduced in diabetic individuals compared to control (n=43; *p<0.05; $$p<0.01) and pre-diabetic (n=43; #p<0.05) individuals. **C:** Decreased *OGA/RPL37A* expression in the diabetic group *vs.* control group (n=41; *p<0.05).

3.1.2 Decreased *OGT* expression accompanies the onset of diabetes

3.1.2.1 OGT expression is able to distinguish between control and pre-diabetic individuals (FPG, ADA criteria)

We next investigated *OGT* transcript levels between our study population characterized according to ADA FPG diagnostic criteria. Diabetic study participants exhibited significantly lower *OGT* expression levels when compared to control individuals, normalized to all three reference genes. *OGT* was attenuated in the diabetic group compared to the control group by: 17.2 ± 2.3% (*GUS*), 23.3 ± 5.6% (*ACTB*); and 26 ± 5.1% (*RPL37A*), respectively (Figure 3.4 A, B, C). In addition, *OGT* was differentially expressed between the control and pre-diabetic groups by 11.5 ± 5.4% (*GUS*) and 17.5 ± 5.5% (*ACTB*) (Figure 3.4 A, B). *RPL37A* normalized data exhibited a decrease in *OGT* expression in the diabetic group *vs.* the pre-diabetic group (19.2 ± 5.6%) (Figure 3.4 C).

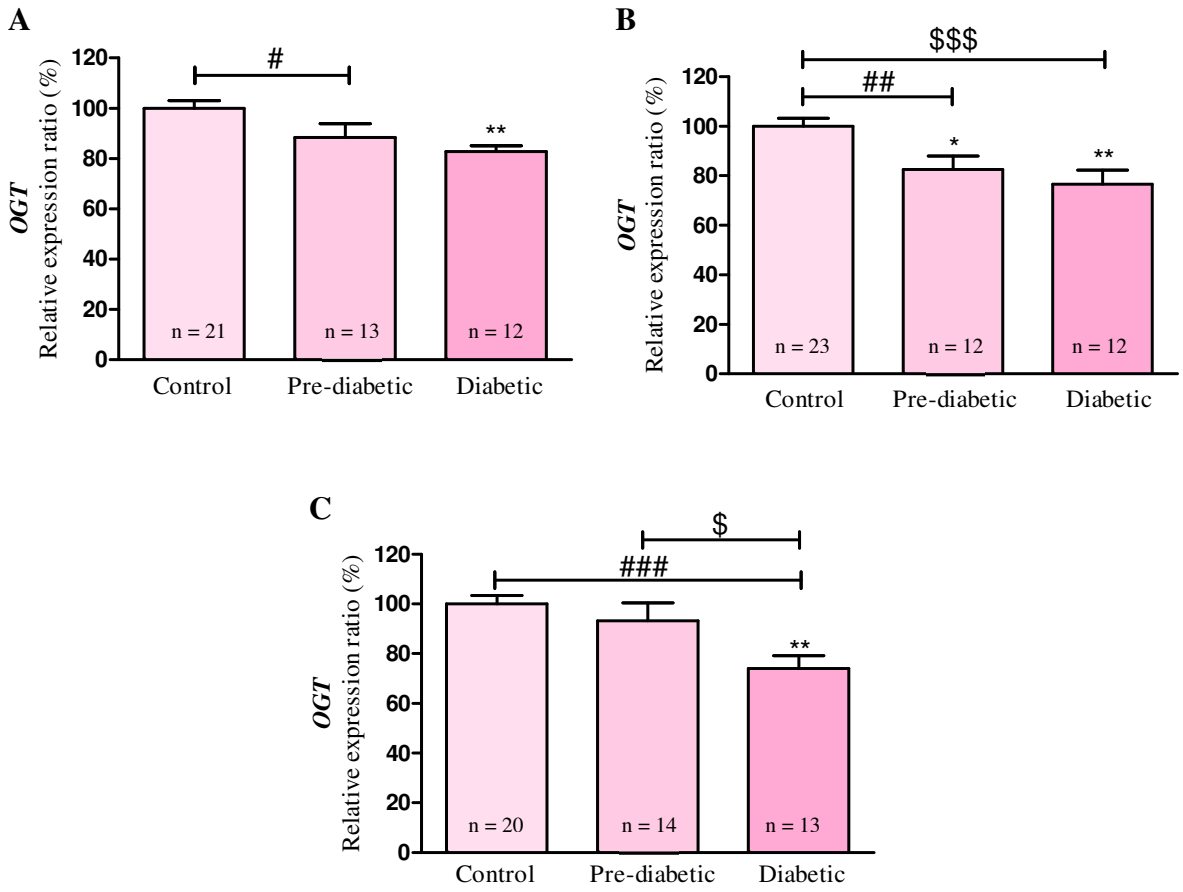

Figure 3. 4. A reduction in *OGT* is accompanied with both pre-diabetes and diabetes (FPG, ADA criteria).

A: Decreased *OGT/GUS* expression in diabetic (n=46; **p<0.01) and pre-diabetic (n=46; #p<0.05) groups compared to the control group. **B:** *OGT/ACTB* is decreased in the pre-diabetic (n=47; **p<0.01; $$$p<0.001) and diabetic (n=47; *p<0.05; ##p<0.01) groups compared to control individuals. **C:** *OGT/RPL37A* levels are attenuated with diabetes compared to controls (n=47; **p<0.01; ###p<0.001) and pre-diabetics (n=47; $p<0.05).

3.1.2.2 OGT expression levels are reduced in diabetic individuals (fasting blood glucose, WHO criteria)

When classed according to FPG (WHO criteria), diabetic volunteers similarly displayed attenuated levels of *OGT* compared to control individuals for all three reference genes. Normalization to *GUS; ACTB* and *RPL37A* resulted in an 18.4 ± 1.8%; 23.2 ± 5.6%; and 24.4 ± 5.3% reduction in *OGT*, respectively (Figure 3.5 A, B, C). As with the ADA criteria, normalization to *ACTB* allowed us to differentiate between control and pre-diabetic

individuals, i.e. a reduction of 19.7 ± 6.3% (Figure 3.5 B). *OGT* normalized to *RPL37A* showed a decrease of 25 ± 5.3% in diabetic volunteers compared to pre-diabetic individuals (Figure 3.5 C).

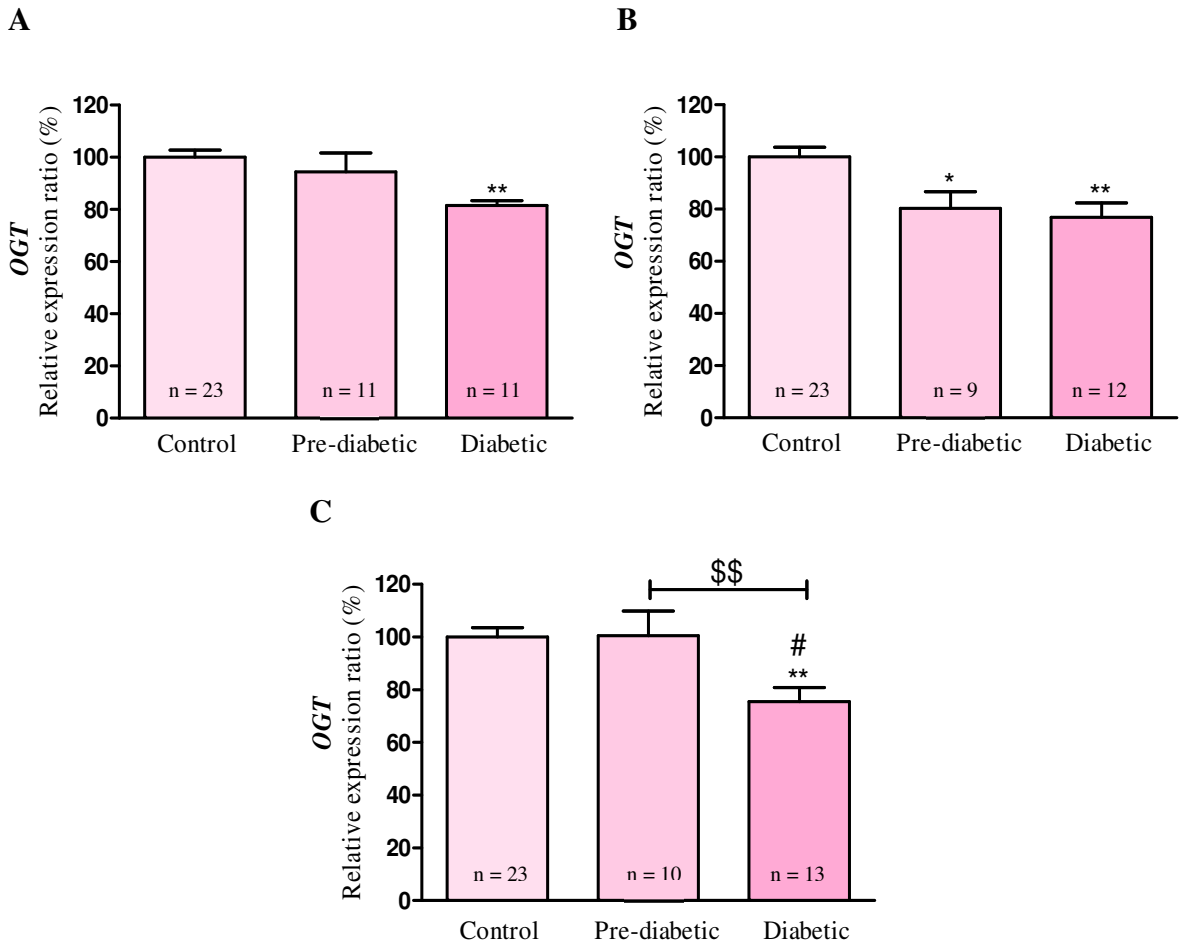

Figure 3. 5. *OGT* expression is diminished in diabetic individuals classed according to FPG levels (WHO criteria).

A: *OGT/GUS* is decreased in diabetic individuals compared to controls (n=45; **p<0.01). **B:** *OGT/ACTB* levels are decreased in diabetics (n=44; **p<0.01) and pre-diabetics (n=44; *p<0.05) compared to control volunteers. **C:** *OGT/RPL37A* expression is attenuated in the diabetic group *vs.* the control (n=46; **p<0.01) and pre-diabetic (n=46; #p<0.05; $$p<0.01) groups.

3.1.2.3 Decreasing OGT expression with rising HbA1c levels (ADA)

When volunteers were characterized using HbA1c levels (ADA criteria), *OGT* expression was again down regulated in diabetic patients *vs.* control individuals. *OGT* normalized to

GUS showed a 23.6 ± 2.2% decrease, while *OGT* normalized to *ACTB* and *RPL37A* presented a 17.8 ± 4.5% and 23.3 ± 3.1% reduction, respectively (Figure 3.6 A, B, C). Also, normalization to *ACTB* allowed us to distinguish between control and pre-diabetic volunteers with a 21 ± 6.7% decrease (Figure 3.6 B). *RPL37A* enabled differentiation between diabetics and pre-diabetics, i.e. we found a 28.8 ± 3% (Figure 3.6 C).

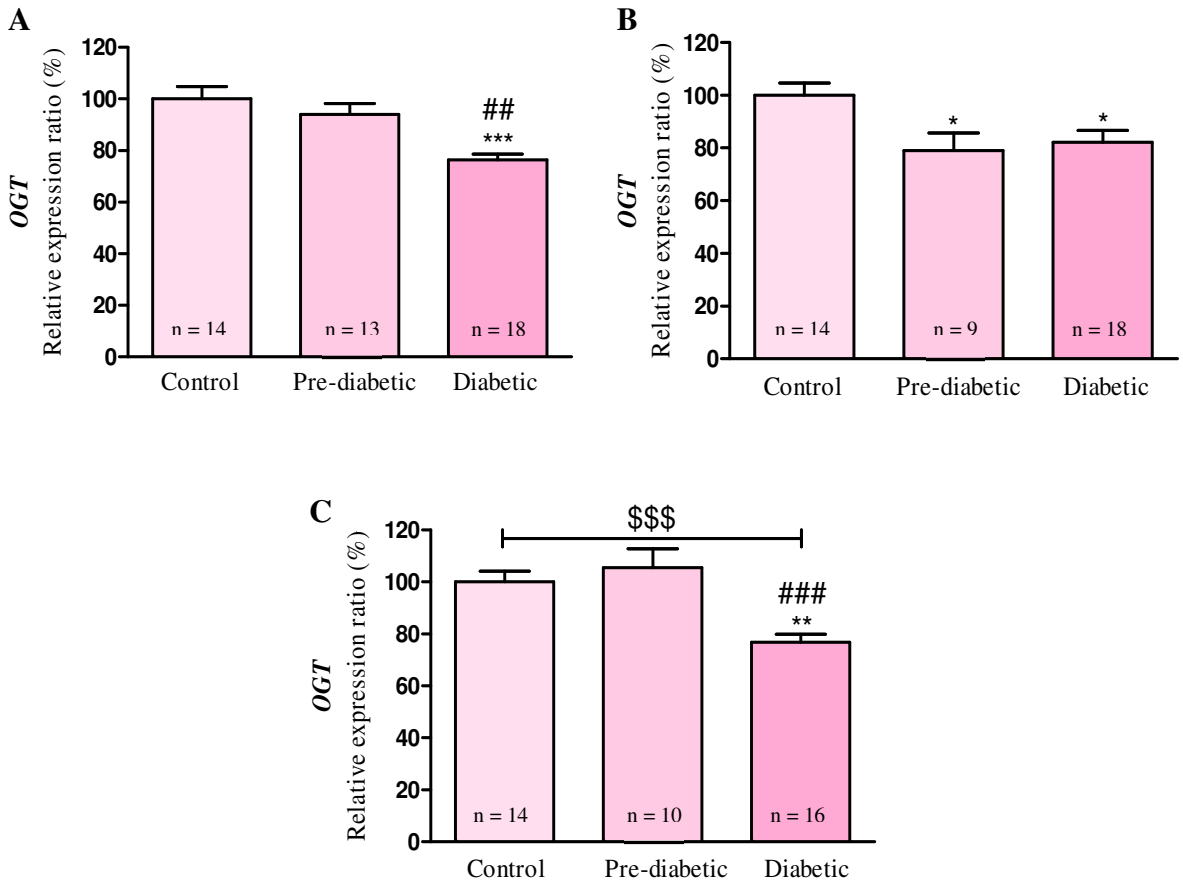

Figure 3. 6. *OGT* expression is attenuated with increasing HbA1c levels (ADA criteria).

A: *OGT/GUS* expression decreases in diabetics *vs.* control (n=45; ***p<0.001) and pre-diabetic (n=45; ##p<0.01) individuals. **B:** *OGT/ACTB* is attenuated with diabetes (n=41; *p<0.05) and pre-diabetes (n=41; *p<0.05) compared to controls. **C:** *OGT/RPL37A* is decreased in diabetic individuals in comparison to control (n=40; **p<0.01; $$$p<0.001) and pre-diabetic (n=40; ###p<0.001) individuals.

3.1.3 Investigation into GFPT expression levels

3.1.3.1 GFPT1 levels remain unchanged with increasing fasting plasma glucose and HbA1c levels, respectively

Due to the relatively low concentration of *GFPT* in our samples, we were compelled to run *GFPT1* and *GFPT2* at a higher cDNA concentration, leaving insufficient amounts of cDNA to normalize to all three reference genes. Hence *GFPT1* and *GFPT2* were only normalized to *GUS* and not to *ACTB* and *RPL37A*.

GFPT1 expression levels revealed no statistically significant differences between any of the three study groups (control, pre-diabetic and diabetic) using all three classification criteria (FPG ADA and WHO, HbA1c ADA).

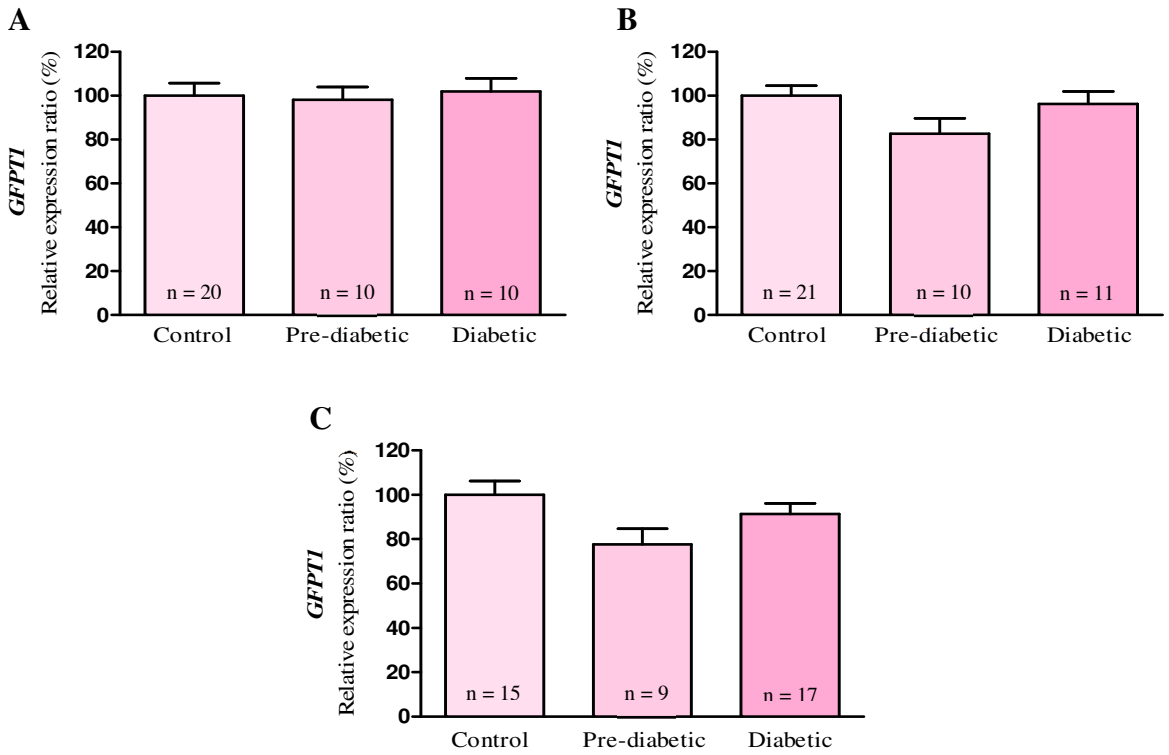

Figure 3. 7. *GFPT1* expression is unaltered with diabetes (all classification criteria).

A: *GFPT1* remains unchanged with increasing fasting plasma glucose levels (n=40) (ADA criteria). **B:** *GFPT1* displays no differential expression between study groups (n=42) (fasting plasma glucose WHO criteria). **C:** Increasing HbA1c levels do not affect *GFPT1* expression levels (n=41) (ADA criteria).

3.1.3.2 GFAT2 expression is unchanged with increasing HbA1c levels (ADA criteria)

Our initial investigations into *GFPT2* expression levels yielded non-significant results between all three study groups, according to fasting plasma glucose levels (ADA and WHO criteria) as well HbA1c levels (ADA criteria) (Figure 3.8 A, B, C).

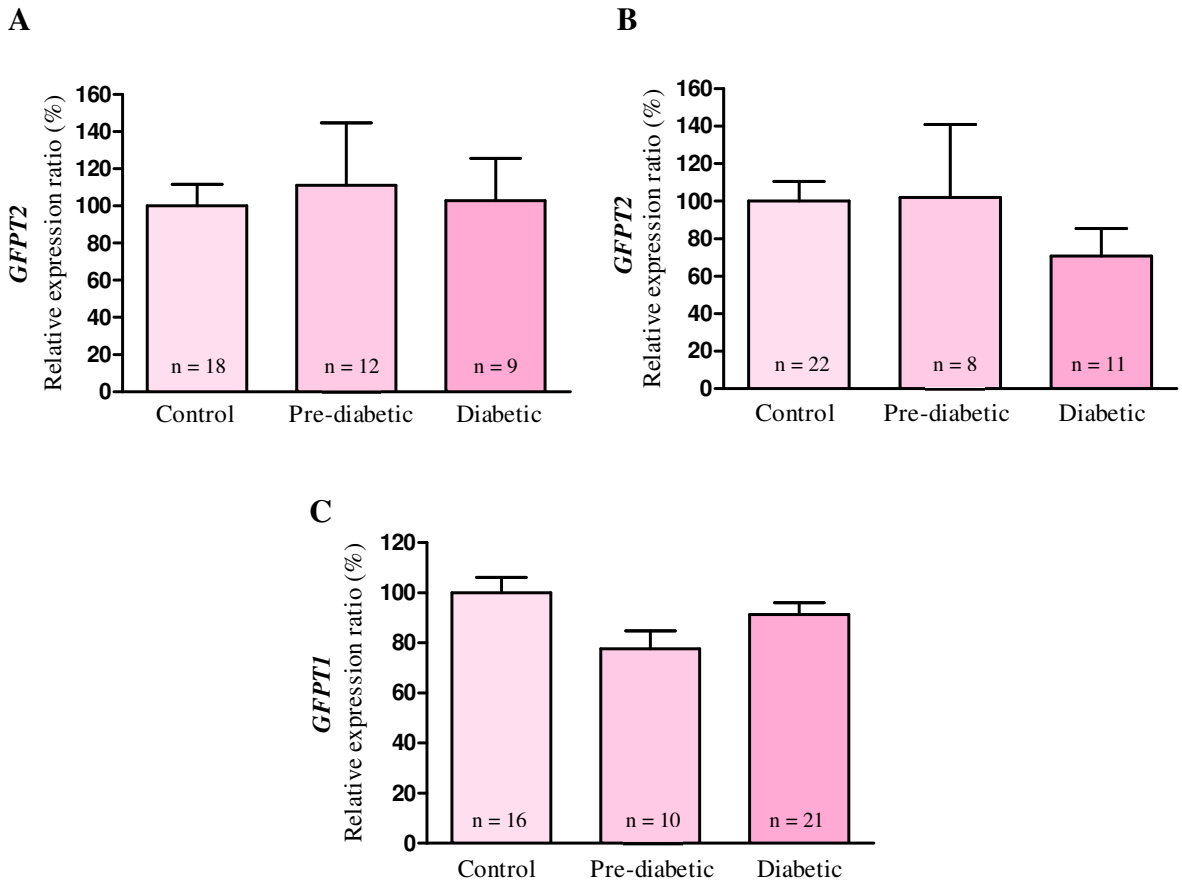

Figure 3. 8. *GFPT2* displays no significantly differential regulation between study groups (all classification criteria).

A: No statistically significant differences are observed in *GFPT2* expression levels with increasing FPG levels (n=39) (ADA criteria). **B:** *GFPT2* is not differentially expressed between study groups (FPG WHO criteria). **C:** *GFPT2* regulation is unaffected by increasing HbA1c levels (ADA criteria).

3.1.3.3 GFAT2 exhibits ethnic-dependent regulation

Upon closer examination of individual *GFPT2* relative expression ratios, we observed a very large inter-individual variability among these ratios (Figure 3.9). The variability appeared to be ethnic related; consequently we decided to separately examine *GFPT2* expression levels for the Caucasian and Mixed Ancestry populations.

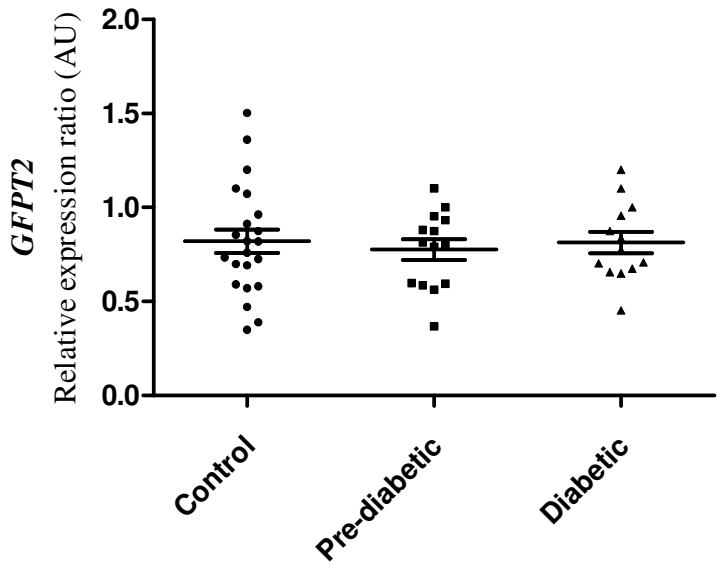

Figure 3. 9. A graphic representation of the large inter-individual variability observed with *GFPT2* expression

Owing to the relatively small number of Mixed Ancestry participants we were unable to examine *GFPT2* expression in this group only; however we were able to do so for the Caucasian population. Here, when participants were classified according to HbA1c levels, we observed a reduction of 54.7 ± 5.3% and 38.8 ± 12.2% in the diabetic and pre-diabetic *vs.* control groups, respectively (Figure 3.10 A). The same trend was observed when patients were characterized according to fasting blood glucose levels (ADA and WHO criteria); however these data were not significant (data not shown due to space constraints). Since differences were only observed when the two participating races were separated, and on

account of large inter-individual variability (that was not seen with any of the other HBP regulatory genes examined in this study), we next investigated differential *GFPT2* expression between Caucasian and Mixed Ancestry participants, regardless of diagnosis. After correcting for diabetic status, our data exposed a 2.4-fold increase in *GFPT2* expression levels in the Mixed Ancestry group compared to the Caucasian group (Figure 3.10B).

It must be noted here that no other ethnic-related differences were found in any of the genes examined in this study (see Figure 3.10 C for an example).

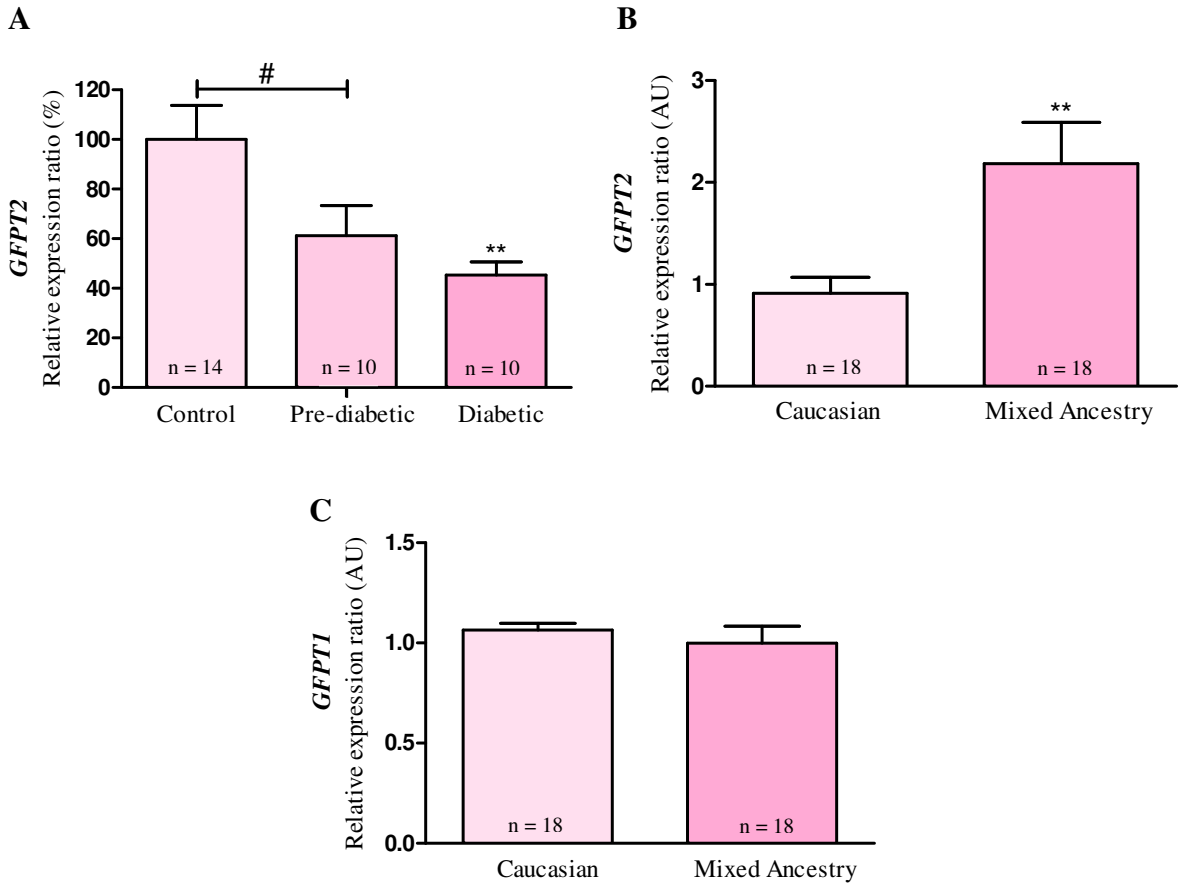

Figure 3. 10. *GFPT2* displays ethnic-dependent regulation whereas other HBP regulatory genes do not (HbA1c classification, ADA criteria).

A: *GFPT2* is decreased in the Caucasian diabetic (n=34; **p<0.01) and pre-diabetic (n=34; #p<0.05) group *vs*. matched controls. **B:** Mixed Ancestry individuals display a 2.4-fold increase in *GFPT2* levels compared to Caucasian individuals, despite diagnosis (n=36; **p<0.01). **C:** *GFPT1* (for example) is not differentially regulated between Mixed Ancestry and Caucasian participants (n=36).

Since GFAT is encoded by two separate genes; *GFPT1* and *GFPT2*, we next investigated differences between our three study groups for *GFPT1* and *GFPT2* levels combined. Since *GFPT2* exhibits ethnic-dependent regulation, we attempted to separately analyze the above in each ethnic group. However, once again the number of Mixed Ancestry participants was inadequate for such analyses. Nevertheless, Caucasian diabetic and pre-diabetic individuals displayed significantly lower expression of *GFPT1* in combination with *GFPT2* (more statistically significant than *GFPT2* levels alone) when characterized according to HbA1c ADA criteria. Diabetic volunteers presented with a marked reduction of 36.3 ± 4.9%, and likewise pre-diabetic individuals by 43.6 ± 6.2% when compared to matched controls (Figure 3.11). When patients were characterized according to fasting plasma glucose levels (ADA and WHO criteria) similar trends were observed, although never reaching statistical significance (data not shown).

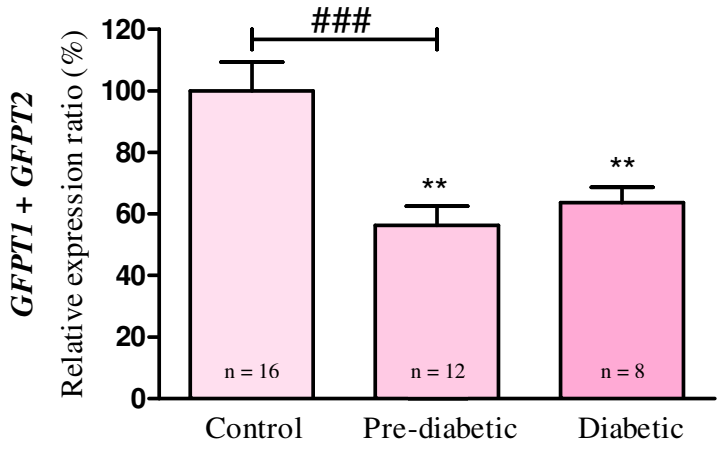

Figure 3. 11. Combined *GFPT* expression decreases in pre-diabetic and diabetic Caucasian individuals (HbA1c, ADA criteria).

(n=33; **p<0.01; ###p<0.001).

3.1.4 *OGT* expression may shed light on discrepancies that exist between FPG and HbA1c classifications

Both the ADA and WHO define diabetes as a FPG of more than 7 mmol/l and an HbA1c level of >6.5%. After critical examination of our cohort, we found that 24.1% of individuals fell into this clear-cut category, while 53.7% of individuals displayed a FPG <7 mmol/l and HbA1c <6.5%. The other 22.2%, however, presented with a FPG <7 mmol/l, but an HbA1c of >6.5%.

Since *OGT* was the most tightly regulated and most significantly differentially expressed HBP regulatory gene, we measured *OGT/GUS* transcript levels between these three groups: 1) >7 mmol/l, >6.5%; 2) <7 mmol/l, <6.5%; and 3) <7 mmol/l, >6.5%. Significant differences were found between the >7 mmol/l, >6.5% and <7 mmol, <6.5%. Encouragingly, *OGT* expression was able to distinguish between individuals where FPG was both <7 mmol/l, but whose HbA1c was either >6.5% or <6.5% (Figure 3.12). Unfortunately, we were not able to assess *OGT's* ability to distinguish between individuals presenting with a FPG of >7 mmol/l in combination with either an HbA1c level of >6.5% or <6.5% as we did not have any volunteers that fell within the >7 mmol/l, <6.5% HbA1c range.

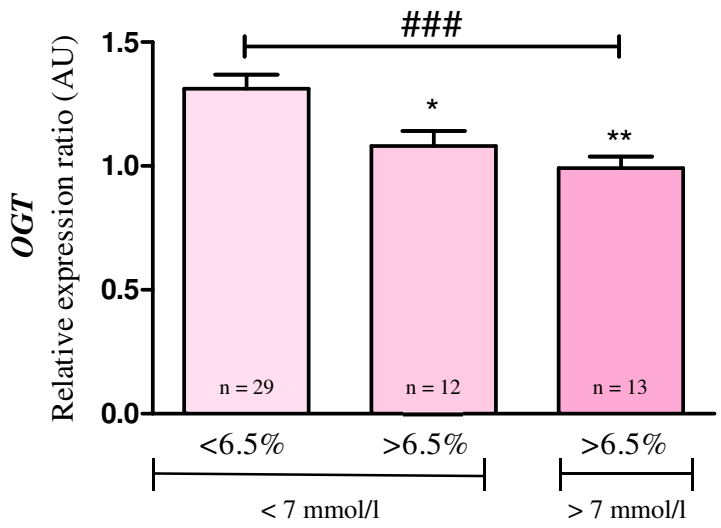

Figure 3. 12. *OGT* expression differs between groups resulting from discrepancies between FPG and HbA1c diagnostic criteria.

(n=54; *p<0.05; **p<0.01; ###p<0.001).

3.2 ASSESSING REGULATION OF THE PPP

mRNA transcript levels were measured using qPCR and SYBR Green 1 chemistry was employed for the detection of gene transcript levels. Study participants were again characterized according to: 1) FPG, ADA criteria; 2) FPG, WHO criteria and 3) HbA1c levels, ADA criteria. Here we measured the expression levels of *TKT* and *TKTL1*; each normalized to *GUS, ACTB* and *RPL37A*.

3.2.1 *TKT* expression essentially remains unchanged

3.2.1.1 TKT expression is unaffected with increasing blood glucose levels (ADA and WHO)

TKT mRNA levels, normalized to all three reference genes, indicated no statistically significant differences in the regulation between control, pre-diabetic and diabetic individuals (FPG, ADA and WHO criteria) (Figure 3.13 A - F).

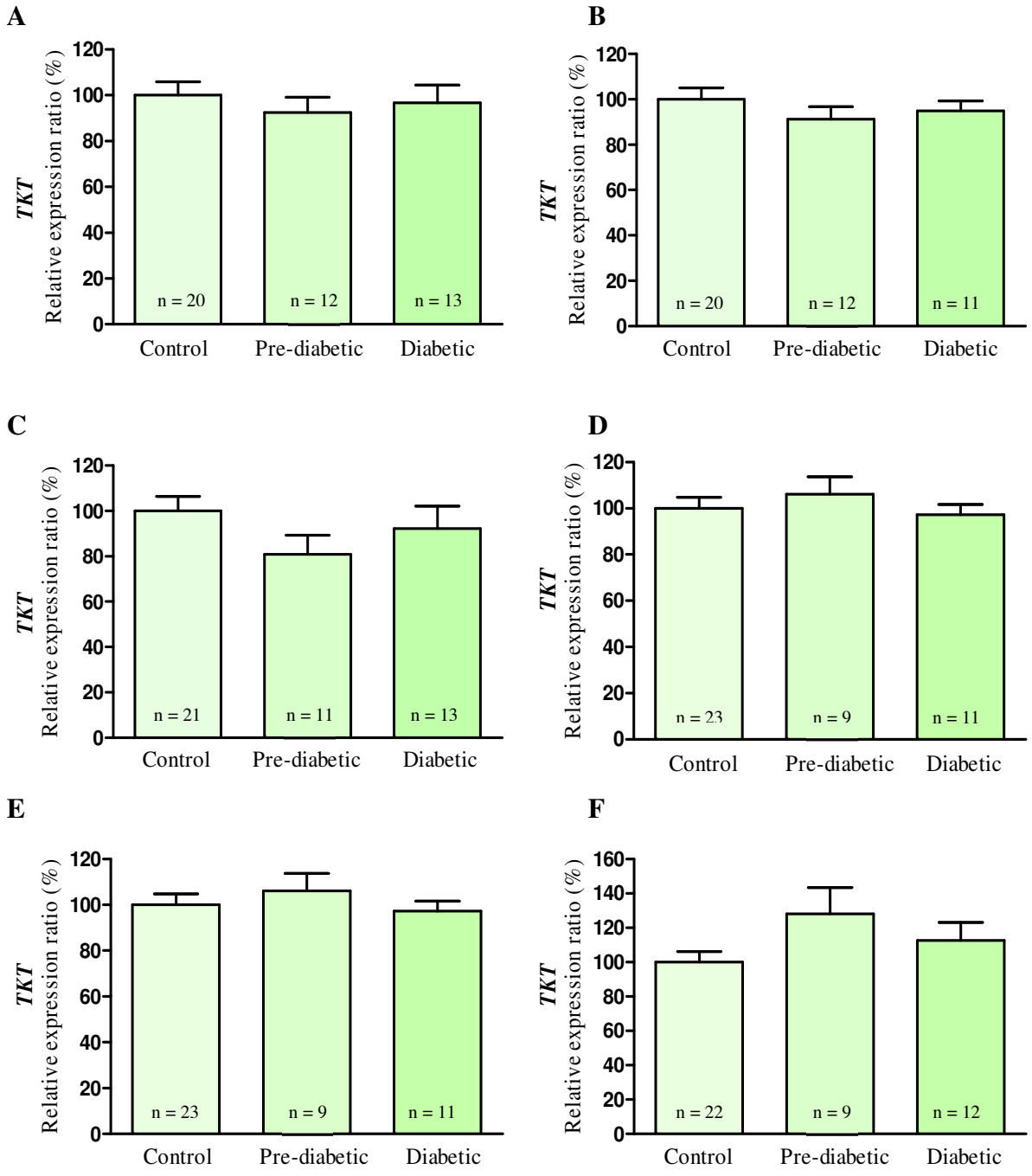

Figure 3. 13. *TKT* is not differentially regulated with increasing fasting blood glucose levels (ADA and WHO criteria).

A: *TKT/GUS* remains constant with increasing plasma glucose levels (n=45) (ADA criteria). **B:** *TKT/ACTB* stays constant with rising plasma glucose levels (n=43) (ADA criteria). **C:** *TKT/RPL37A* is unchanged by increasing blood glucose concentrations (n=45) (ADA criteria). **D:** *TKT/GUS* is unaffected by diabetes diagnosis (n=43) (WHO criteria). **E:** *TKT/ACTB* expression is unaffected by rising blood glucose levels (n=43) (WHO criteria). **F:** *TKT/RPL37A* is unchanged in diabetes (n=43) (WHO criteria).

3.2.1.2 TKT expression may be differentially expressed with increasing HbA1c levels (ADA)

However, when participants were re-characterized according to HbA1c levels, *TKT* expression levels normalized to *GUS* and *RPL37A* were decreased by 22.9 ± 5.3% and 31 ± 4.9%, respectively, between the diabetic group compared to the pre-diabetic group (Figure 3.14 A, C).

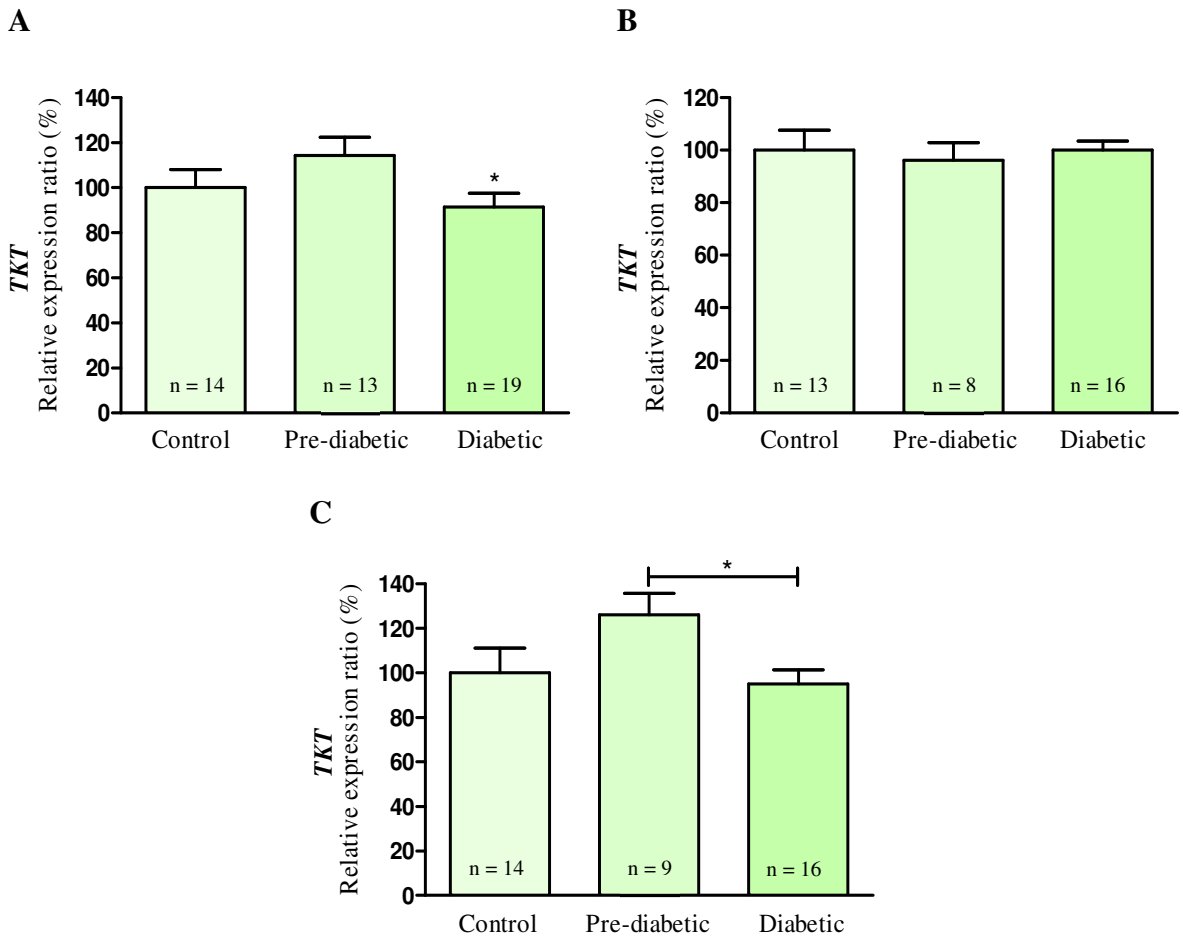

Figure 3. 14. HbA1c levels indicate a difference in *TKT* expression between diabetic and pre-diabetic individuals (ADA criteria).

A: *TKT/GUS* expression level is decreased is diabetic individuals compared to controls (n=46; *p<0.05). **B:** *TKT/ACTB* remains constant between study groups with increasing HbAlc levels (n=37). **C:** *TKT/RPL37A* expression is attenuated from pre-diabetes to diabetes (n=39; *p<0.05).

3.2.2 *TKTL1* is differentially expressed with diabetes

3.2.2.1 TKTL1 levels are augmented with pre-diabetes and diabetes (FPG, ADA criteria)

TKTL1 expression levels were markedly increased in diabetic individuals, characterized according to fasting blood glucose (ADA criteria), compared to healthy controls. *TKTL1* expression demonstrated an increase of: 98.6 ± 44.3%; 74.7 ± 37.2%; and 73.4 ± 43.7% in the diabetic *vs.* control group for all three reference genes: *GUS; ACTB* and *RPL37A,* respectively (Figure 3.15 A, B, C). In addition, *TKTL1* expression levels were also elevated in pre-diabetic individuals compared to controls. Normalization to *GUS* revealed an 86 ± 30.1% increase in *TKTL1* in pre-diabetics; whereas normalization to *RPL37A* showed *TKTL1* levels to be increased by 73 ± 22.9% in the pre-diabetic group when compared to control individuals (Figure 3.15 A, C).

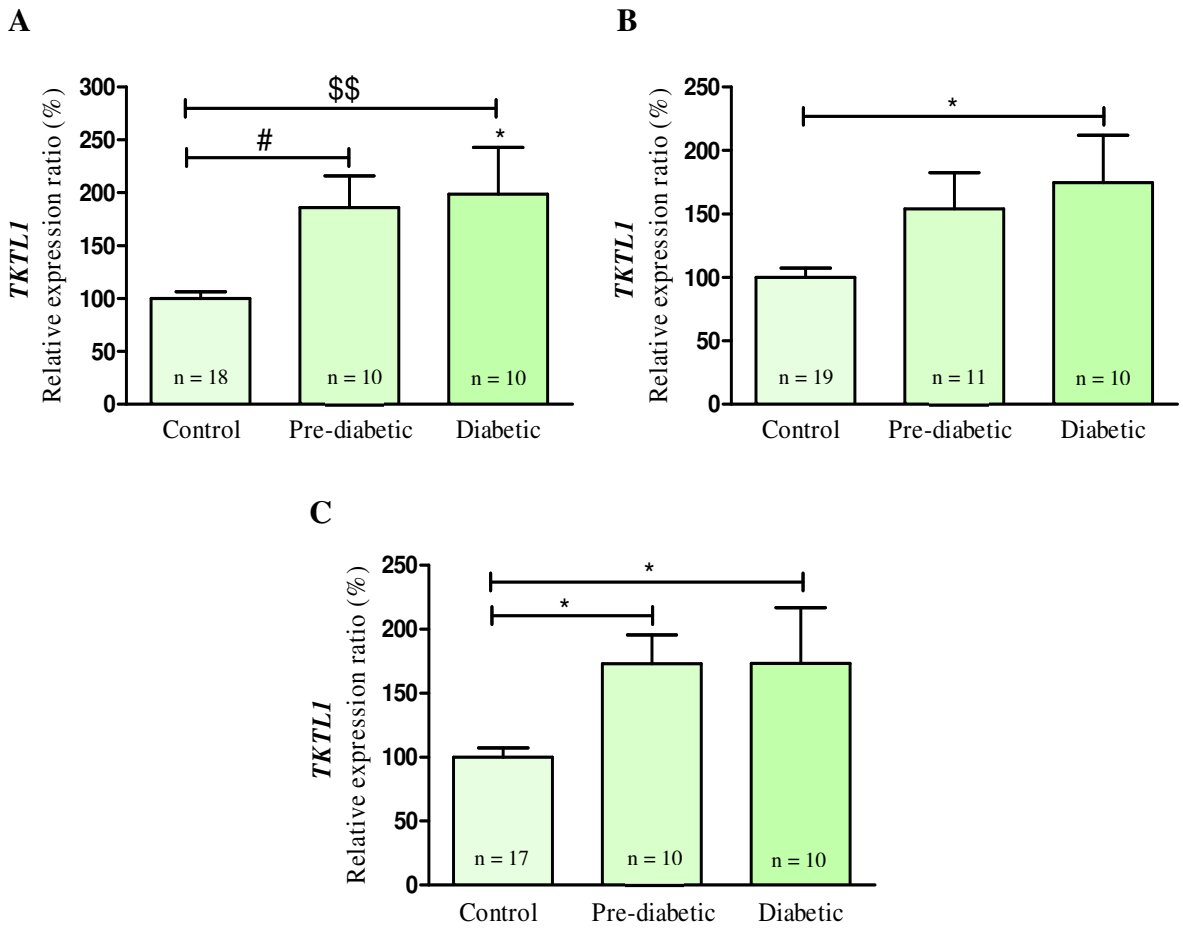

Figure 3. 15. *TKTL1* expression increases in parallel with higher fasting blood glucose levels (ADA criteria).

A: Diabetic (n=38; *p<0.05; $$p<0.01) and pre-diabetic (n=38; #p<0.05) groups show elevated levels of *TKTL1/GUS* expression *vs.* controls. **B:** *TKTL1/ACTB* is increased in diabetic individuals compared to controls (n=40; *p<0.05). **C:** *TKTL1/RPL37A* is augmented in diabetic (n=37; *p<0.05) and pre-diabetic (n=37; *p<0.05) groups when compared to control individuals.

3.2.2.2 WHO classification alters the observed significance of TKTL1 up regulation

When volunteers were re-characterized according to the WHO criteria for FPG, specifying a pre-diabetic category of 6.1 - 6.9 mmol/l (*vs.* the ADA's more conservative 5.6 - 6.9 mmol/l), the observed statistical significance was largely lost. Despite trends remaining similar, only normalization to *GUS* revealed a statistically significant increase in *TKTL1* expression levels (64.3 ± 36.7%) in the diabetic group when compared to control individuals (Figure 3.16 A).

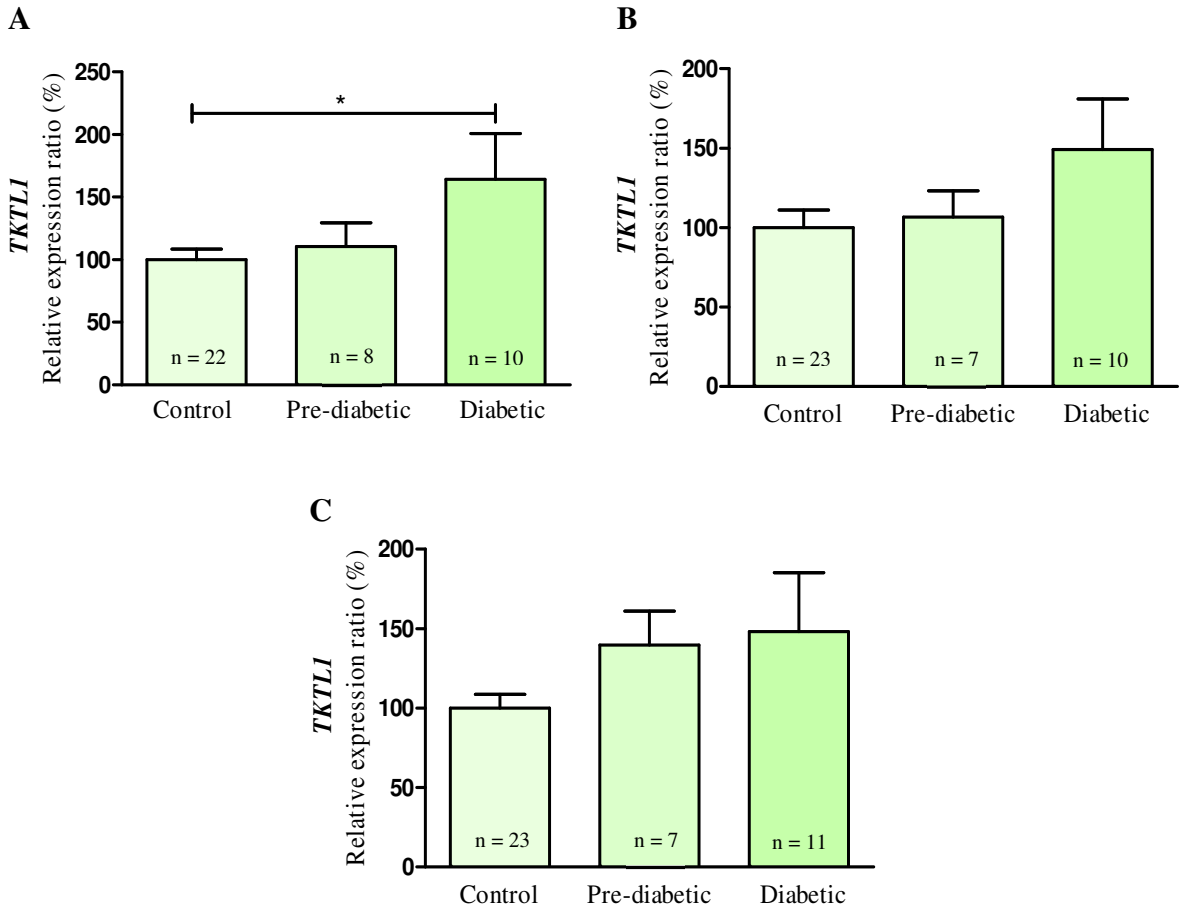

Figure 3. 16. *TKTL1* is largely unchanged with increasing FPG levels (WHO criteria).

A: *TKTL1/GUS* levels are increased in diabetic volunteers compared to healthy counterparts (n=40; *p<0.05). **B:** *TKTL1/ACTB* is not differentially expressed with diabetes diagnosis (n=40). **C:** *TKTL1/RPL37A* is not significantly altered by increasing blood glucose levels (n=41).

3.2.2.3 HbA1c characterization fails to significantly enhanced TKTL1 with diabetes

Despite the increase of 58.1 ± 28% in *TKTL1* expression in the diabetic group *vs.* the control group and 84.5 ± 38.2% *vs.* the pre-diabetic group (normalized to *ACTB*) (Figure 3.17B); as with the WHO fasting plasma glucose criteria, *TKTL1* upregulation with diabetes is no longer statistically significant when characterized according to HbA1c levels (ADA criteria) (Figure 3.17 A, C).

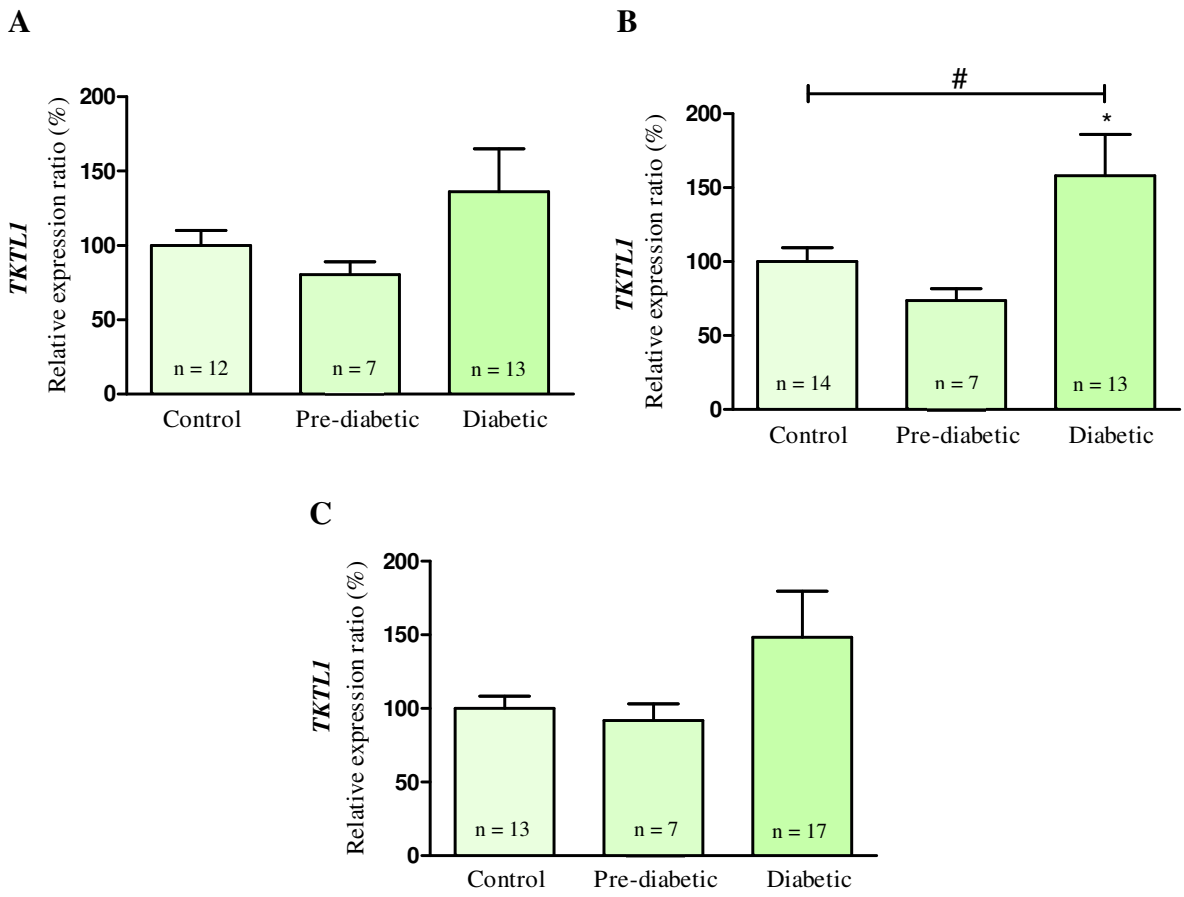

Figure 3. 17. HbA1c levels, for the most part, do not alter *TKTL1* expression (ADA criteria).

A: *TKTL1/GUS* is not significantly altered between study groups (n=32). **B:** *TKTL1/ACTB* is upregulated in the diabetic group compared to control individuals (n=34; *p<0.05). **C:** *TKTL1/RPL37A* shows no significant differences between groups (n=37).

The large SEM between study groups (indicated graphically by the use of error bars) indicates that *TKTL1* relative expression ratios were grouped closely together for the control group (represented graphically by a smaller error bar); but as glucose dysregulation increased (with increasing fasting plasma glucose), *TKTL1* expression levels became more widely dispersed (Figure 3.18).

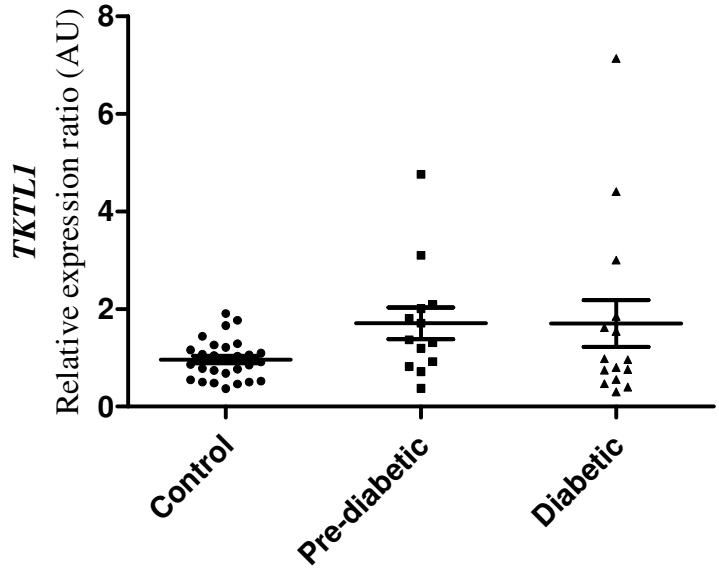

Figure 3. 18. *TKTL1* inter-individual variability increases with rising blood glucose concentrations (ADA criteria).

3.3 REGULATION OF THE POLYOL PATHWAY WITH DIABETES

3.3.1 The polyol pathway may be differentially regulated with the onset of pre-diabetes and diabetes

3.3.1.1 AKR1B1 expression is unaffected by rising blood glucose levels (ADA)

Examination of *AKR1B1* transcript levels revealed no significant differences between our study groups when normalized to all reference genes (Figure 3.19).

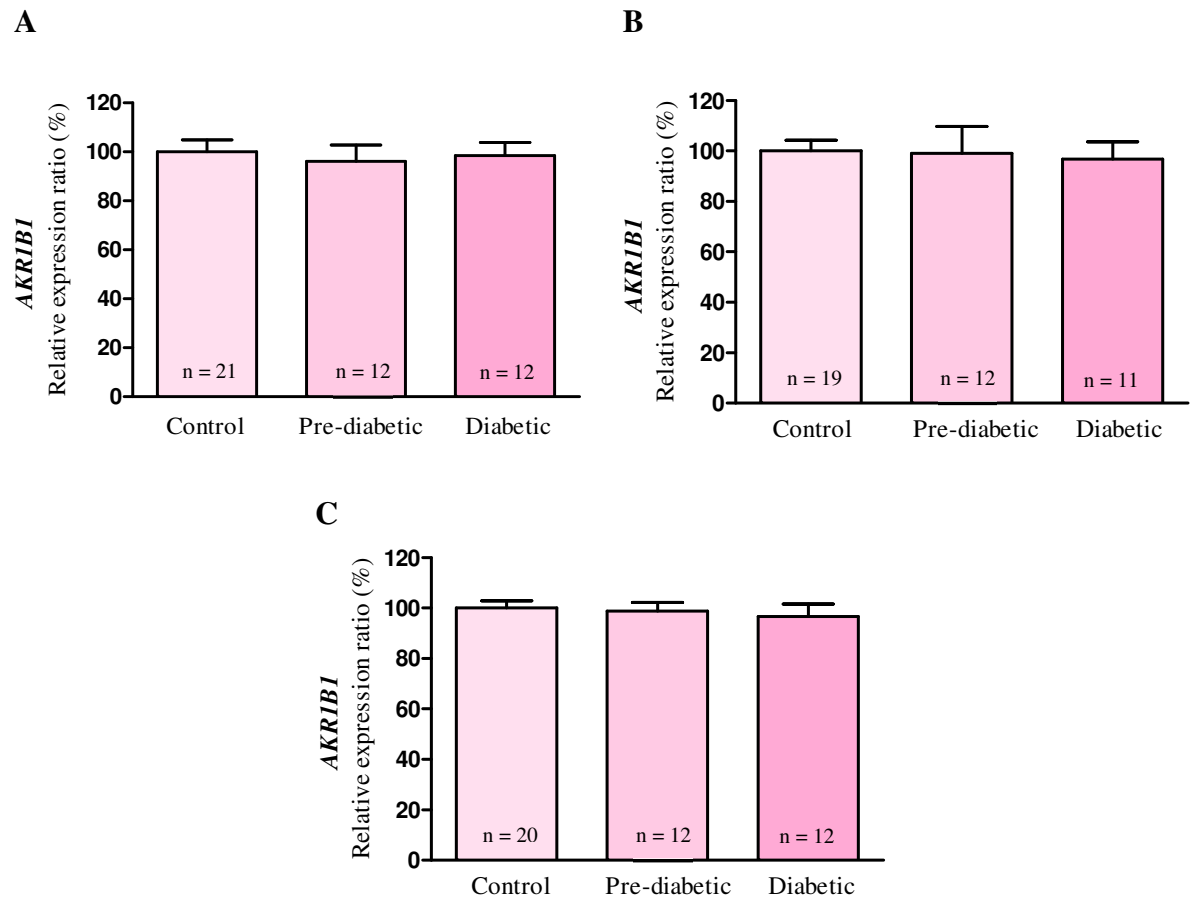

Figure 3. 19. Increasing blood glucose levels have no effect on *AKR1B1* expression (ADA criteria).

A: *AKR1B1/GUS* is not differentially expressed between study groups (n=45). **B:** *AKR1B1/ACTB* expression remains constant between groups (n=42). **C:** *AKR1B1/RPL37A* is not significantly different between controls, pre-diabetics and diabetics (n=44).

3.3.1.2 AKR1B1 levels are attenuated with pre-diabetes (FPG, WHO and HbA1c, ADA)

However, when study participants were re-characterized according to WHO criteria, *GUS* and *ACTB* normalized data showed a decrease in *AKR1B1* expression levels with the onset of pre-diabetes. *AKR1B1* was decreased by 21.5 ± 4.9% (*GUS* normalized) and by 30.9 ± 5.1% (*ACTB* normalized) in the pre-diabetic group versus control individuals (Figure 3.20 A, B). However, data normalized to *RPL37A* indicated no significant differences between the three groups (Figure 3.20 C).

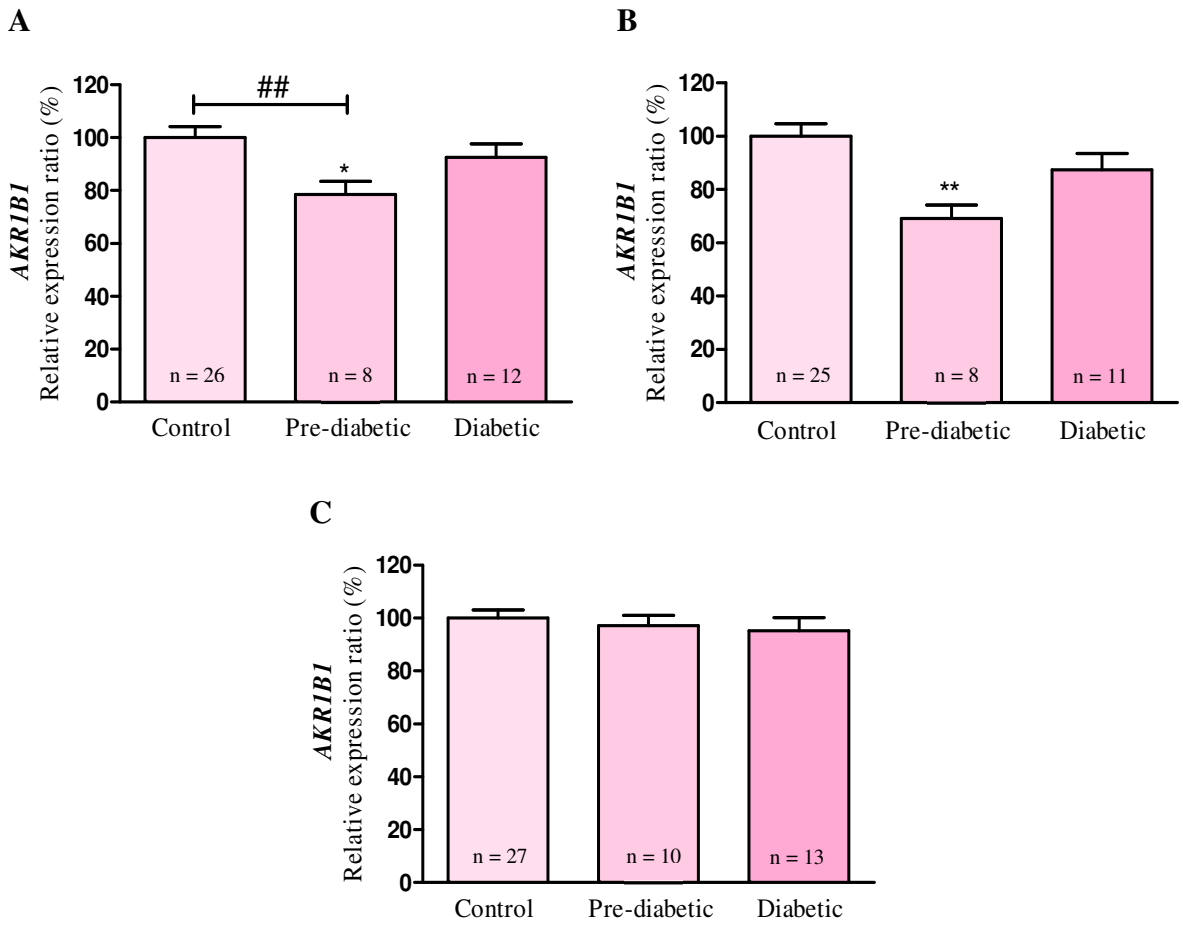

Figure 3. 20. *AKR1B1* levels are attenuated with the onset of pre-diabetes (FPG WHO criteria).

A: *AKR1B1/GUS* is decreased in pre-diabetic individuals *vs.* controls (n=46; *p<0.05). **B:** *AKR1B1/ACTB* expression is reduced in the pre-diabetic group when compared to the control group (n=42; **p<0.01). **C:** *AKR1B1/RPL37A* is not differentially expressed with diabetes (n=50).

The same changes were observed when patients were characterized according to HbA1c levels (ADA criteria), although here we observed a slight decrease in *AKR1B1* expression levels (18.1 ± 4.6%) in the diabetic group when compared to controls, for *GUS* normalized data (Figure 3.21 A). *ACTB* normalized data showed a difference of 28.9 ± 6.8% between the control and pre-diabetic groups (Figure 3.21 B). Again, *ARK1B1* normalized to *RPL37A* yielded no significant differences between the groups (Figure 3.21 C).

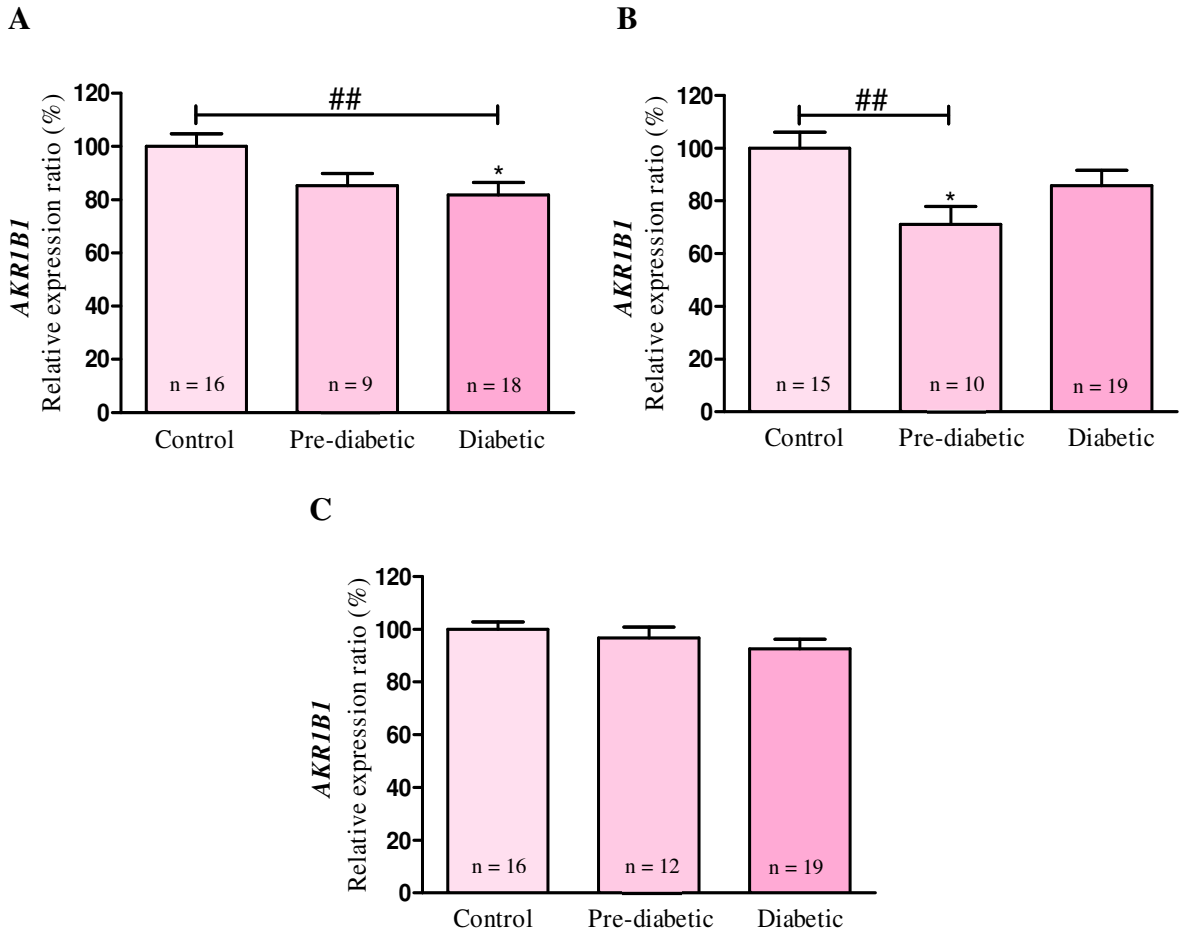

Figure 3. 21. Varying expression of *AKR1B1* with increasing HbA1c levels (ADA criteria).

A: *AKR1B1/GUS* is decreased in the diabetic group when compared to controls (n=43; *p<0.05; ##p<0.01). **B:** *AKR1B1/ACTB* is reduced in pre-diabetic *vs.* control individuals (n=44; *p<0.05; ##p<0.01). **C:** *AKR1B1/RPL37A* is not differentially expressed between groups (n=47).

Chapter 4

DISCUSSION

Even with the current diagnostic tools at our disposal, diabetes remains largely under-diagnosed (22). As discussed, there are several aspects that hinder the accurate and successful diagnosis of diabetes including: biological shortcomings of each assay (41); a lack of standardization in diagnosis due to alternative diagnostic approaches (41) and the incongruity between organization-specific cut-off criteria (28). Since diabetes and other non-communicable diseases have reached epidemic proportions (7) and the consequences thereof are both detrimental to the individual and the economy (19), exploration into novel diagnostic tools for the earlier detection of diabetes is a reasonable initiative. Hyperglycemia-induced flux through alternative NOGPs is strongly linked to the development of insulin resistance and diabetes-associated pathologies (105). Consequently, we hypothesized that the genes encoding the regulatory enzymes of these respective pathways may be differentially expressed between control, pre-diabetic and diabetic individuals, so representing an exploratory and novel diagnostic avenue, based on genetic biomarkers, for the earlier recognition of type 2 diabetes.

The conclusions drawn from this study are:

1) *OGA* expression decreases in diabetic subjects
2) Pre-diabetic and diabetic individuals present with decreased *OGT* expression levels
3) *GFPT2* expression exhibits ethnic-dependent regulation
4) *GFPT1* mRNA levels are not significantly altered with diabetes
5) *TKT* expression does not vary significantly between study groups
6) Pre-diabetic and diabetic subjects display increased levels of *TKTL1*
7) *AKR1B1* expression may potentially be decreased with the onset of pre-diabetes

4.1 *OGA* EXPRESSION DECREASES IN DIABETIC SUBJECTS

OGA encodes the protein *O*-GlcNAcase which is responsible for the removal of *O*-GlcNAc moieties from target proteins (178). Although normalizing to three separate reference genes and different characterizations yielded varying degrees of sensitivity, the same fundamental trend was observed: decreased *OGA* expression in diabetic persons.

Since increased *O*-GlcNAcylation of target proteins accompanies diabetes and OGA is responsible for the removal of *O*-GlcNAc moieties, our finding of decreased *OGA* expression levels in diabetic individuals was not surprising. All three classification groups (i.e. FPG, ADA criteria; FPG, WHO criteria and HbA1c, ADA criteria) demonstrated a decrease in *OGA* expression for the diabetic group compared to control individuals (Figure 3.1 A, B; Figure 3.2 A, B, C; Figure 3.3 A, B, C). Moreover, when patients were characterized according to WHO criteria pre-diabetic individuals also presented with attenuated *OGA* levels compared to controls, thus potentially allowing for the earlier detection of type 2 diabetes (Figure 3.2 A, B). These data visibly illustrate how *OGA* sensitivity is affected when participants are differently characterized, thereby emphasizing the consequences of conflicting diagnostic thresholds. This is a trend that is observed throughout the Results section.

The outcome of decreased *OGA* expression is consistent with earlier clinical studies presenting increased *O*-GlcNAcylation with type 2 diabetes (43) (159). Therefore, lower gene expression of the enzyme responsible for the removal of *O*-GlcNAc moieties in diabetic individuals may support the reported elevation in *O*-GlcNAcylation. Furthermore, laboratory studies have described the manifestation of insulin resistance/ type 2 diabetes with the use of *O*-GlcNAcase inhibitors (164). Additional support for decreased *OGA*/ *O*-GlcNAcase

expression with diabetes comes from a study carried out on a Mexican American population. Here researchers found that a mutation in the *OGA* gene resulted in the early termination of *OGA* translation leading to decreased expression and increased susceptibility to type 2 diabetes (67). The finding of decreased *OGA* corroborates previous work carried out in our laboratory where we found reduced leukocyte OGA protein expression in diabetic patients characterized according to HbA1c levels (189). Here patients characterized according to FPG levels (ADA and WHO criteria) displayed the same trend; however only apparent when employing additional selection criteria, i.e. diabetics with a fasting plasma glucose level of >9 mmol/l. This suggests that variations in expression at the gene level may be more sensitive than the protein level when examining OGA levels.

Conversely, there have also been reports of increased OGA expression with human type 2 diabetes, although on the protein level. For example, Park *et al.* (2010) reported augmented OGA protein expression in erythrocytes of diabetic individuals (43). Likewise, in the study previously carried out in our laboratory when OGA protein expression levels were measured in leukocyte sub-populations (i.e. granulocytes and lymphocytes), diabetic individuals now presented with augmented OGA levels (159). Both groups proposed that this may be an adaptive response in order to decrease high levels of *O*-GlcNAcylation, thereby restoring function to the cell and avoiding the damaging effects of hyper-*O*-GlcNAcylation.

We propose that the incongruities observed between the studies discussed above and the present one may stem from a variety of sources. It is our opinion that the specific cell type under examination (erythrocytes *vs.* total leukocyte population *vs.* granulocytes and lymphocytes) may greatly impact on the observations since different cell types may contain varying numbers of *O*-GlcNAc modifiable proteins and exhibit distinct *O*-GlcNAc cycling

rates (159). In addition, differences in population admixture may affect observations. Moreover, it has been proposed that due to the highly complex regulation of both *OGA* and *OGT*, mRNA levels may not necessarily correlate with what is observed on the protein level (160). Regardless of such inconsistencies, our data reveal a timely and significant decrease in *OGA* expression levels which supports the notion of increased *O*-GlcNAcylation with type 2 diabetes and provides impetus for further investigation.

4.2 PRE-DIABETIC AND DIABETIC INDIVIDUALS PRESENT WITH DECREASED *OGT* EXPRESSION LEVELS

O-GlcNAc transferase is encoded by the *OGT* gene and is ubiquitously expressed and highly conserved among species (170) (171). OGT is responsible for the dynamic addition of *O*-GlcNAc moieties onto serine/ threonine residues of target proteins (146).

Since previous studies reported increased *O*-GlcNAcylation with the progression of type 2 diabetes (43) (159), we anticipated increased expression of the gene encoding the enzyme responsible for the addition of *O*-GlcNAc molecules to target proteins in diabetic individuals. Our data pointed to a statistically robust and early decrease in *OGT* expression levels (Figure 3.4 A, B, C; Figure 3.5 A, B, C; Figure 3.6 A, B, C). When study recruits were classed according to FPG (ADA criteria), pre-diabetic and diabetic individuals exhibited reduced *OGT* expression compared to the control group (Figure 3.4 A, B). This finding, as with *OGA*, highlights the effects that organization-specific diagnostic thresholds may have on sensitivity. However, this result was surprising and raises the question how to reconcile attenuated *OGT* expression with higher *O*-GlcNAcylation typically found with type 2 diabetes. We suggest that this subtle, yet significant decrease in *OGT* expression at the pre-diabetic and diabetic

stage may represent an adaptive response in order to decrease the number of *O*-GlcNAcylated proteins under hyperglycemic conditions and consequently minimize the damaging effects associated with hyper-*O*-GlcNAcylation. Since it is well recognized that the *O*-GlcNAc modification rapidly responds to external stimuli such as an increase in nutrients in order to maintain protein stability, activity and function; it is reasonable to assume that cells would attempt to decrease *O*-GlcNAcylation in order to maintain proper cell functionality (146). Of note, diabetic patients displayed greater reductions in *OGA* expression relative to *OGT* suggesting that despite an attempt to lessen *O*-GlcNAcylation (by reducing *OGT* expression) and restore balance, the reduction in *OGA* expression outweighs the proposed adaptive response and could ultimately result in increased global *O*-GlcNAcylation, characteristic of diabetes.

Conversely, others (including our research group) found unchanged levels of OGT in diabetic individuals, albeit on the protein level. However, our research group experienced difficulty in quantifying OGT levels due to limitations of the antibody employed and hence the data should be interpreted with caution (159) (189). The other study that examined OGT levels in erythrocytes (43) employed Western blotting as the primary method for OGT detection. This is a more qualitative method and certainly less quantitative than qPCR, the method used in this study. In addition to the variability that may stem from the use of different techniques, the complex regulation of OGT could also impact on the variability observed between the protein and gene level (160). In addition to regulation by UDP-GlcNAc concentrations, OGT also readily undergoes PTM that is regulated by a wide variety of kinases and can itself be *O*-GlcNAcylated (163). As a result, changes at the mRNA level may not directly correlate with protein expression/ enzyme activity. Moreover, *OGT* is alternatively spliced to produce three distinct isoforms localized to different tissues/ cellular compartments (168) and investigation

into isoform expression levels should also be analyzed when commenting on total OGT expression. All the complexities discussed above need to be deliberated when examining *OGT* fluctuations in response to hyperglycemia as OGT defies regulation by a single mechanism and consequently many factors may impact upon its expression level.

Both the ADA and WHO define diabetes as a FPG of more than 7 mmol/l and an HbA1c level of >6.5% (26). After critical examination of our cohort, we found that a relatively large number of volunteers (22.2%) presented with a FPG <7 mmol/l, but an HbA1c of >6.5%, resulting in a conflict of diagnosis and further highlighting the discordances that exist between glucose based assays and HbA1c testing. A recent study also encountered this issue with up to 78% of their study cohort falling into this category (190). Such discrepancies have serious repercussions and can potentially lead to misdiagnosis if either the glucose-based assay or HbA1c assay are used in singularity. Unfortunately the use of a single diagnostic test is often a reality due to financial constraints (49).

We consequently attempted to determine if *OGT* expression levels could differentiate these conflicting groups from one another. Further investigation into the diagnostic capability of *OGT* revealed that expression levels were able to distinguish between those individuals who presented with a FPG level of <7 mmol/l, but who exhibited either an HbA1c of >6.5% or <6.5% (Figure 3.12). Such findings further increase the potential of *OGT* expression levels in accurately diagnosing type 2 diabetes and reflect its ability to define changes in glycemia.

4.3 *GFPT1* mRNA LEVELS ARE NOT SIGNIFICANTLY ALTERED WITH DIABETES

Together, *GFPT1* and *GFPT2* encode for GFAT, the rate-limiting enzyme of the HBP (181) (182). *GFPT1* expression was unaffected by increasing FPG (ADA and WHO criteria) and HbA1c levels (Figure 3.7 A, B, C), nor did it display ethnic-related differences at baseline (Figure 3.10 C). *GFPT1* is primarily regulated on a post-transcriptional level (191) and so we were not surprized to not find any statistically significant differences in *GFPT1* expression with increasing blood glucose concentrations or HbA1c levels. It is likely that *GFPT1* may be post-transcriptionally and/ or post-translationally regulated and hence unaltered mRNA levels would not necessarily confer unchanged enzyme activity/ protein expression. In support, Robinson *et al.* (2007) measured *GFPT1* mRNA levels and enzyme activity in diabetic rats and found that while enzyme activity was substantially down-regulated, *GFPT1* mRNA levels remained constant, implying post-transcriptional regulation (192). Thus while *GFPT1* mRNA levels are not significantly altered between control, pre-diabetic and diabetic individuals the enzyme activity may be differentially regulated between these groups. We therefore recommend measuring enzyme activity and protein expression levels in parallel with mRNA levels when assessing overall GFAT expression in response to diabetes.

4.4 *GFPT2* EXPRESSION EXHIBITS ETHNIC-DEPENDENT REGULATION

Initially we observed no significant changes in *GFPT2* expression (Figure 3.8 A, B, C) according to FPG and HbA1c classifications. However, the large inter-individual variability we observed with *GFPT2* expression levels (Figure 3.9) prompted us to separately examine expression levels for the two participating races. Due to the relatively small sample size we were unable to perform this analysis for the Mixed Ancestry group alone. However,

Caucasian pre-diabetic and diabetic individuals displayed a marked decrease in *GFPT2* expression levels compared to control individuals (HbA1c characterization) (Figure 3.10 A).

Since GFAT regulates HBP flux (129) and increased *O*-GlcNAcylation is associated with type 2 diabetes (43) (159), we were surprized to find attenuated levels of *GFPT2* in pre-diabetic and diabetic individuals. Furthermore, our data are in disagreement with others who reported higher *GFPT2* mRNA expression levels in diabetic individuals from the Indian subcontinent (184). Such inconsistencies may be due to the technique employed to measure *GFPT2* expression, e.g. the Indian study used semi-quantitative PCR that is known to have several methodological limitations when compared to qPCR. In addition, these researchers examined *GFPT2* levels in a leukocyte sub-population (lymphocytes) whereas we measured expression in the total leukocyte population. This once again highlights differences dependent on the cell type investigated and is a phenomenon we feel is very important when assessing HBP flux and regulation. Measuring HBP expression levels in the total leukocyte population as well as in leukocyte sub-populations may help to clarify this concept and allow for further comment. Since different cell types contain varying amounts of *O*-GlcNAc modifiable proteins with distinct cycling rates, it is rational to suppose that expression of HBP regulators may vary accordingly (159). *GFPT2* mRNA down-regulation may also not necessarily correlate with enzyme activity and protein expression levels since *GFPT2* undergoes post-translational modifications (157) (193). However, since GFAT is negatively regulated by UDP-GlcNAc (151), and UDP-GlcNAc is the substrate for the *O*-GlcNAc modification (which is upregulated with diabetes), the observed decrease in *GFPT2* expression in pre-diabetic and diabetic individuals may be in response to increased levels of UDP-GlcNAc with hyperglycemia.

The concept of ethnic-dependent regulation is further supported by our finding that *GFPT2* expression levels demonstrated a 2.4-fold increase in the Mixed Ancestry population compared to Caucasian individuals, regardless of diagnosis (Figure 3.10 B). These data are consistent with another study where it was established that African-Americans exhibit a 2-fold increase in *GFPT2* expression compared to Caucasian individuals (194). Increased *GFPT2* expression levels in the Mixed Ancestry population may also help explain the relatively high prevalence of type 2 diabetes in this population residing within the greater Cape Town region (Western Cape, South Africa) (195).

Since both *GFPT1* and *GFPT2* encode the enzyme GFAT and the most statistical significance was observed with HbA1c characterization, we evaluated their combined expression level with increasing HbA1c levels.

Here we found that the combined expression was significantly decreased in both Caucasian pre-diabetic and diabetic individuals, to a larger extent than *GFPT2* alone (Figure 3.11). The enhanced statistical significance may be explained by the fact that *GFPT1* is not primarily regulated at a transcriptional level and hence differences in *GFPT1* expression (minor decrease in pre-diabetic and diabetic individuals, but not statistically significant) alone may not have reached statistical significance (Figure 3.7 C), but when combined with *GFPT2* the collective statistical significance was enhanced (Figure 3.11).

4.5 *TKT* EXPRESSION DOES NOT VARY SIGNIFICANTLY BETWEEN STUDY GROUPS

Transketolase is encoded by the *TKT* gene and is the rate-limiting enzyme of the non-oxidative branch of the PPP (140) (142). A key function of the PPP is to produce the antioxidant NADPH that can be used to scavenge harmful ROS and hence this pathway is proposed to be beneficial during the onset and progression of diabetes (106), although this remains controversial (138) (139). This "protective" role is further acknowledged due to the ability of the PPP to shunt glycolytic intermediates into pentose reactions, thereby diverting flux through potentially damaging pathways (134).

Our data reveal no statistically significant changes in *TKT* expression with increasing FPG levels using both ADA and WHO diagnostic criteria (Figure 3.13 A - F). These data are supported by current work in our laboratory that show no change in TKT protein expression levels with acute hyperglycemia (equivalent to pre-diabetes) in cell culture (Joseph DE and Essop MF, unpublished data). *TKT* mRNA expression levels alone may not be an accurate reflection of flux through the PPP, as a recent study found that diabetic individuals presented with a thiamine deficiency, an important co-factor for TKT, when compared to non-diabetic subjects (196). Hence flux through the PPP may additionally be affected by thiamine levels and this too should be considered when assessing PPP flux in response to hyperglycemia/ type 2 diabetes. Furthermore, two additional TKT-like enzymes (TKTL1 and TKTL2) (141) (142) that have proposed transketolase activity may form hetrodimers with TKT impacting on total transketolase activity and hence the flux through the PPP (197).

When participants were characterized according to HbA1c levels, diabetic patients exhibited reduced *TKT* levels in comparison to pre-diabetic individuals (Figure 3.14 A, C). Characterization according to FPG levels revealed no statistically significant changes in *TKT* expression (Figure 3.13 A - F), while HbA1c levels did. Since diabetes is clinically defined

by increased blood concentrations (measured by FPG) and not by the extent of glycated proteins (measured by HbA1c levels), the two assays are proposed to identify different groups of diabetic individuals (40). Moreover, HbA1c measure the average glycemia over a three-month period compared to FPG that measures daily fluctuations in glycemia suggesting that HbA1c may provide a more stable, specific measurement of hyperglycemia (40). It is for these reasons that we may be observing a statistically significant difference in *TKT* expression levels when employing HbA1c diagnostic criteria compared to FPG criteria.

This reduction may represent an "exhausted" PPP in diabetic individuals. Flux through the PPP may be increased in pre-diabetic individuals in an initial attempt to generate NADPH to aid in the scavenging of accumulating hyperglycemia-induced ROS. Eventually, the damaging effects of the alternative damaging pathways may outweigh the beneficial effects of the PPP, driving the body into a state of full-blown diabetes and further promoting flux through the harmful pathways with a consequential decrease through the PPP. These interesting concepts require further investigations to test whether this is indeed the case.

4.6 PRE-DIABETIC AND DIABETIC SUBJECTS DISPLAY INCREASED LEVELS OF *TKTL1*

In addition to TKT, there are two additional human transketolase-like proteins, TKTL1 and TKTL2 (142). TKTL1 is encoded by the *TKTL1* gene that shares 61% sequence identity with *TKT* (198) and has recently received much attention in the literature due to the controversial debate regarding its proposed transketolase activity. Whereas some groups accept that TKTL1 possesses transketolase activity (141), Schneider *et al.* (2012) demonstrated the

converse with an elegantly designed deletion study (143). Therefore this issue remains unresolved and further studies are required to shed light on the precise function of TKTL1.

While our findings do not in any way resolve this debate, they clearly (and to our knowledge for the first time) show that *TKTL1* is differentially regulated with type 2 diabetes. Our data reveal that *TKTL1* expression levels are significantly increased with rising blood glucose concentrations and can differentiate between control and pre-diabetic individuals as well as control and diabetic patients (ADA criteria) (Figure 3.15 A, B, C). However, when patients were characterized according to WHO criteria the observed significance was largely lost (Figure 3.16 A, B, C), demonstrating once again the impact of using different diagnostic thresholds. When employing the WHO's reference range for pre-diabetes (FPG level of 6.1-6.9 mmol/l), many ADA classified pre-diabetics now fall into the control group. Thus the sensitivity between control and pre-diabetic categories is lost with WHO characterization when compared to the more conservative characterization of the ADA (pre-diabetes: 5.6 - 6.9 mmol/l).

If TKTL1 truly does possess transketolase activity then we could assume that the PPP is up regulated with increasing FPG (ADA criteria). Since a previous study reported that targeted knockdown of *TKTL1* caused a >50% reduction in total transketolase activity (199), this indicates that *TKTL1* has a regulatory effect on total transketolase activity and thus it could mean that increased *TKTL* (with higher FPG) may reflect greater PPP flux, despite unchanged *TKT* levels. However, additional studies at the enzyme activity level are required to resolve this intriguing issue.

It is well known that the PPP is responsible for the generation of NADPH, a key antioxidant. However, the PPP is also necessary for fatty acid metabolism (94) that is usually increased

with diabetes (12). Thus another possibility is that the upregulation of the PPP may occur with diabetes in order to support increased fatty acid metabolism. Higher PPP flux may also elicit additional metabolic effects. Since the PPP does not generate ROS (as the other damaging pathways do), the PPP may represent a safer, "ROS-free" way to metabolize surplus glucose in diabetic individuals (200). Together the upregulation of the PPP during hyperglycemia/ type 2 diabetes may support an increase in FFA metabolism (indicative of type 2 diabetes) and also possibly a "safer" alternative to metabolize surplus glucose. However, we propose that this protective capacity of the PPP is not sufficient to outweigh the harmful effects induced by the four damaging NOGPs and hence diabetes-associated complications will eventually arise (106).

We found that *TKTL1* inter-individual variability was lowest in the control group and higher in the pre-diabetic group while further increasing in the diabetic group (Figure 3.18). Whether this has implications is unknown as nothing was found in the literature to corroborate this finding, although it is interesting to note. If *TKTL1* does not in fact possess transketolase activity it may be safe to assume that whatever the function is, it is certainly linked to increased glucose metabolism. This is supported by the finding that *TKTL1* mRNA expression is increased in many tumor cells when compared to healthy cells (201). Aggressive cancer cells often consume 20 - 30% more glucose than the average cell and accordingly, like type 2 diabetes, also represent a state of increased glucose metabolism (200). Moreover, increased *TKTL1* expression seems to be generally harmful, e.g. others found negative correlations with increased *TKTL1* expression (e.g. higher *TKTL1* correlated with poor patient prognosis (202) and increased metastases (145)). To sum up we have shown, for the first time as far as we are aware, that *TKTL1* is markedly increased in pre-diabetic and diabetic individuals when compared to healthy controls (ADA criteria),

confirming a role in increased glucose metabolism. Thus *TKTL1* gene expression analysis has potential as a novel diagnostic marker to help detect pre-diabetes and full-blown type 2 diabetes.

4.7 *AKR1B1* EXPRESSION MAY POTENTIALLY BE DECREASED WITH THE ONSET OF PRE-DIABETES

AKR1B1 codes for the enzyme aldose reductase that catalyzes the conversion of toxic aldehydes to inactive alcohols in the first step of the polyol pathway (116). We found that *AKR1B1* expression levels remained unchanged between control, pre-diabetic and diabetic volunteers with increasing FPG concentrations (ADA criteria) (Figure 3.19 A, B, C).

AKR1B1 is predominantly expressed in tissues prone to diabetic complications (e.g. retinal cells) hence its strong association with diabetes-associated micovascular pathologies (116). For example, previous studies have found variants in the *AKR1B1* gene to be linked with diabetes-related complications such as retinopathy, nephropathy and neuropathy (203). Several studies have also shown *AKR1B1* gene and protein expression to be elevated in type 2 diabetic patients suffering from microvascular complications (204) (205) (206).

When participants were characterized according to WHO criteria pre-diabetic individuals had reduced levels of *AKR1B1* when compared to control individuals. This finding remains unclear and a better patient history with consequent alternate classifications may help to clarify this finding. When study participants were characterized according to HbA1c levels, no consensus could be reached between the three respective reference genes and hence we are unable to comment on *AKR1B1* expression in response to rising HbA1c levels (Figure 3.21 A, B, C). To sum up, the likelihood of observing differential *AKR1B1* gene expression is

much higher in diabetic individuals suffering from microvascular complications. Therefore we propose that it has poor diagnostic utility when employing leukocytes isolated from pre-diabetic and full-blown diabetic persons.

4.8 DIAGNOSTIC UTILITY OF GENE EXPRESSION IN TYPE 2 DIABETES

Despite the identification of >40 type 2 diabetes risk loci, together they only account for ~5 - 10% of the well-established heritability observed with type 2 diabetes (54) . Accordingly, genetic variants may be less effective in tracking the progression of a disease where many of the commonly found "risk" genes only account for small effects. It has therefore been suggested that not only alterations in genomic sequences may confer disease susceptibility, but that altered gene expression may also play an important role (64). In light of this, it is our opinion that altered gene expression may prove useful to assist with the diagnosis of type 2 diabetes.

Our central hypothesis is that genes involved in the regulation of the HBP (such as *OGA* and *OGT*) would prove valuable since the endpoint of the pathway (*O*-GlcNAcylation) is upregulated with diabetes. The latter is widely implicated in the regulation of gene transcription, both directly through its association with RNA polymerase II (essential for catalyzing DNA transcription) and indirectly by modifying a host of transcription factors (extensively reviewed in (146)). *O*-GlcNAcylation is also nutrient-sensitive and therefore it acts as a significant link between environmental cues and the development of type 2 diabetes. In support, there is robust evidence that strongly suggests that *O*-GlcNAcylation plays a dynamic part in nutrient-responsive gene expression through its ability to regulate transcription (207). Dysregulated *O*-GlcNAcylation in response to hyperglycemia therefore

likely initiates changes in transcription (initially short-lived and reversible), but with prolonged and chronic hyperglycemia may become permanent and result in dysfunction (146). In light of these factors we are of the opinion that the HBP is a worthy candidate when exploring novel diagnostic avenues for type 2 diabetes.

4.9 APPRAISAL OF THE METHOD EMPLOYED

In order to determine differential gene expression in our cohort we employed the use of qPCR. We believe that qPCR is highly sensitive, quantifiable, reproducible, and time savvy and a high-throughput method that could be translated into a clinical setting, thereby lending diagnostic appeal (187). Moreover, there is room for improvement on the current study since enhanced detection chemistries that confer greater accuracy and specificity such as fluorescent probes (e.g. FAM versus SYBR Green I used in this study) could be employed (208). Fluorescent probes also allow for multiplex PCR, i.e. the assessment of more than one target gene in a single reaction well thereby enabling the assessment of a number of target genes in a limited time period (208). The sensitivity of detection may also be improved with the use of different reference genes. For the current study we employed three distinct reference genes that did not always yield the same statistical significance, but more reference genes should be evaluated in order to achieve the most accurate readings. Discrepancies such as these indicate that while the use of reference genes is the gold standard in qPCR (186), other methods such as linear regression of efficiency (LRE) (209) (less sensitive to inter-individual variability) should be explored when attempting to normalize expression data as this could further enhance the potential of the current study. However, it is important to consider the cost of qPCR when proposing this method as an exploratory diagnostic avenue for diabetes. While it remains relatively costly, current diagnostic tests have all been

introduced, revised and amended over decades and consequently the same could be expected with the inception of a new diagnostic tool allowing time for the costs associated with qPCR to decrease (24).

Despite the relative limitations of SYBR Green I technology (e.g. essential to use highly specific primer pairs as SYBR green binds to all double-stranded DNA) and the use of reference genes, we still managed to obtain statistically sound data and with the scope for methodological improvements we believe that differential gene expression has potential in the search for new diagnostic tools for the earlier and more efficient detection of type 2 diabetes.

4.10 LIMITATIONS OF THE STUDY

Inevitable to research, this study was not without limitations and included the following:

1) Although the use of reference genes is unavoidable and considered the gold standard in qPCR, our data clearly demonstrate that different reference genes yield varying degrees of sensitivity. The use of more than three reference genes would have been ideal to validate our results, but this was practically not possible due to time constraints, limited volumes of sample cDNA, and budgetary constraints.

2) The ideal study cohort would have consisted of newly diagnosed patients who had not yet been exposed to glucose-lowering medications and exogenous insulin treatment as such treatment regimens may affect our results. However, due to the difficulty in obtaining type 2 diabetic volunteers such requests were unattainable.

3) A larger study population consisting of equal amounts of Caucasian and Mixed Ancestry individuals would have allowed us to determine the effects of ethnicity on gene expression. Although this was done in the Caucasian population, there were insufficient numbers of Mixed Ancestry volunteers from each group (control, pre-diabetic and diabetic) to determine differential gene expression in this group alone.

4) Gender, HIV status and prescribed medications were not taken into account when assessing differential gene expression.

5) An important limitation to consider in this study is that the initial characterization of our study population is based on methods that are discordant and subject to their respective limitations, e.g. FPG is subject to large intra-individual variability.

   Thus it is possible that a number of volunteers may have been "incorrectly" characterized as only a once-off FPG reading was available for FPG ADA characterization. Moreover, although volunteers were reminded to arrive in a fasted state, some admitted to consuming beverages such as coffee or tea a few hours prior to assessment. Since the study guarantees patient anonymity we were unable to identify these patients retrospectively. The generation of false positives may also have resulted in mis-characterization that may ultimately skew our gene expression data by de-sensitizing differences between study groups.

4.11 FUTURE RESEARCH RECOMMENDATIONS

To further advance this study we would recommend the following:

1) In order to validate the current findings and further investigate differential gene expression as a potential diagnostic tool a much larger sample size is needed. This would also allow for the determination of the effects of gender and ethnicity on gene expression.

2) To assess the effects of glucose-lowering medications and insulin treatment on gene expression.

3) Investigate whether specimen collection in a non-fasted state would yield the same promising results as this is an appealing characteristic of a diagnostic tool.

4) Include the measurement of enzyme activity and protein expression levels for the genes under investigation.

5) To evaluate gene and protein expression, as well as the corresponding enzyme activity in different leukocyte subpopulations. Since more statistically robust protein data was previously obtained by our research group in granulocytes and lymphocytes versus total leukocytes, this may represent an opportunity to further strengthen the statistical significance observed in this study.

6) While differential expression of HBP regulators shows great promise in diagnosing type 2 diabetes, an alternative route would be to examine the expression of OGT's binding partners whose expression it is known to alter, particularly those involved in insulin signaling since it remains the key metabolic derangement in type 2 diabetes.

4.12 CONCLUSION

This preliminary study into the differential expression of key regulatory genes involved in alternative non-oxidative glucose metabolism shows reasonable potential. The early and significantly robust changes in HBP biomarkers is of particular interest due to its ability to detect the onset of both pre-diabetes and overt type 2 diabetes, and may offer diagnostic value in the future.

This study also highlighted the need for investigation into such exploratory diagnostic avenues since current assays are discordant and discrepancies between organization-specific cut-off criteria greatly impact on the successful and accurate diagnosis of diabetes. Here our *OGT* data was able to discriminate between such discrepancies. Moreover, we believe that due to the tightly regulated, reversible, highly responsive, and metabolically integrating nature of the *O*-GlcNAc modification; gene expression in response to nutritional status (as regulated by *O*-GlcNAcylation) may represent a more stable, exceptionally sensitive measurement of average glucose fluctuations. We have also shown, for the first time, that *TKTL1* is differentially expressed with pre-diabetes and overt type 2 diabetes and that despite its controversial function, it appears to play a role in increased glucose metabolism. This pilot study into the investigation of alternative NOGPs highlights the link between regulatory gene expression and altered glucose metabolism/ type 2 diabetes. The HBP and its biomarkers have clearly emerged as candidates that warrant further investigation into its potential use in the fight against the growing global diabetes epidemic.

Chapter 5

REFERENCES

(1) Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010 Jan;87(1):4-14.

(2) Federation ID. Global Diabetes Plan 2011-2021. 2011. Available from: http://www.idf.org/global-diabetes-plan-2011-2021 (cited 6 March 2012).

(3) Pradeepa R, Prabhakaran D, Mohan V. Emerging economies and diabetes and cardiovascular disease. Diabetes Technol Ther 2012 Jun;14 Suppl 1:S59-67.

(4) Alwan A. Global status report on noncommunicable diseases 2010. : World Health Organization 2011 April.

(5) Colagiuri R, Keeling A. United Nations High Level Meeting on Non-Communicable Diseases: An unprecedented opportunity. Diabetes Res Clin Pract 2011;93(2):137-138.

(6) Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004 May;27(5):1047-1053.

(7) Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011 Dec;94(3):311-321.

(8) Hall V, Thomsen RW, Henriksen O, Lohse N. Diabetes in Sub Saharan Africa 1999-2011: epidemiology and public health implications. A systematic review. BMC Public Health 2011 Jul 14;11:564.

(9) Mbanya JC, Motala AA, Sobngwi E, Assah FK, Enoru ST. Diabetes in sub-Saharan Africa. The Lancet 2010 Jun 26;375(9733):2254-2266.

(10) Kalra S, Kalra B, Agrawal N, Unnikrishnan A. Understanding diabetes in patients with HIV/AIDS. Diabetol Metab Syndr 2011 Jan 14;3(1):2.

(11) Faurholt-Jepsen D. The double burden. Dan Med J 2013 Jul;60(7):B4673.

(12) Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006 Dec 14;444(7121):840-846.

(13) Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond) 2008 Sep;32(9):1431-1437.

(14) Patel KC, Bhopal R. Diabetes epidemic in the South Asian Diaspora: action before desperation. J R Soc Med 2007 Mar;100(3):115-116.

(15) Mohanty SA, Woolhandler S, Himmelstein DU, Bor DH. Diabetes and cardiovascular disease among Asian Indians in the United States. J Gen Intern Med 2005 May;20(5):474-478.

(16) Nesto RW. Correlation between cardiovascular disease and diabetes mellitus: current concepts. Am J Med 2004 March;116(Supplement 5A):S11-S22.

(17) Ruta LM, Magliano DJ, LeMesurier R, Taylor HR, Zimmet PZ, Shaw JE. Prevalence of diabetic retinopathy in Type 2 diabetes in developing and developed countries. Diabetic Med 2013;30(4):387-398.

(18) Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev 2013 Jan;93(1):137-188.

(19) Gambert SR, Pinkstaff S. Emerging Epidemic: Diabetes in Older Adults: Demography, Economic Impact, and Pathophysiology. Diabetes Spectrum 2006;19(4):221-228.

(20) Clark CM, Fradkin JE, Hiss RG, Lorenz RA, Vinicor F, Warren-Boulton E. Promoting early diagnosis and treatment of type 2 diabetes. JAMA: the journal of the American Medical Association 2000;284(3):363-365.

(21) Sherwin RS, Anderson RM, Buse JB, Chin MH, Eddy D, Fradkin J, et al. The prevention or delay of type 2 diabetes. Diabetes Care 2004 Jan;27(Supplement 1):S47-S54.

(22) King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 1998 Sep;21(9):1414-1431.

(23) UKPDS Group. UK Prospective Diabetes Study 6. Complications in newly diagnosed type 2 diabetic patients and their association with different clinical and biochemical risk factors. Diabetes Res 1990;13(1):1-11.

(24) Cox ME, MD, Edelman D, MD. Tests for Screening and Diagnosis of Type 2 Diabetes. Clinical Diabetes 2009;27(4):132-138.

(25) Historical vignette" honey urine" to pancreatic diabetes: 600 BC-1922. Mayo Clinic proceedings. Mayo Clinic; 1971 Jan;46(1):56-58.

(26) Diagnosis and classification of diabetes mellitus. Diabetes Care 2013 Jan;36(1):S67-S74.

(27) Gillett MJ. International expert committee report on the role of the A1c assay in the diagnosis of diabetes: diabetes care 2009; 32(7):1327–1334.

(28) Mollar-Puchades MA, Pallarés-Carratalá V, de Solís MSN, Piñón-Sellés F. Fasting Glucose Versus Oral Glucose Tolerance Testing in the Diagnosis of Diabetes Mellitus. Rev Esp Cardiol 2006;59(12):1349-1350.

(29) Singh B, Saxena A. Surrogate markers of insulin resistance: A review. World journal of diabetes 2010;1(2):36.

(30) Kim SH, Chunawala L, Linde R, Reaven GM. Comparison of the 1997 and 2003 American Diabetes Association classification of impaired fasting glucose: impact on prevalence of impaired fasting glucose, coronary heart disease risk factors, and coronary heart disease in a community-based medical practice. J Am Coll Cardiol 2006 Jul 18;48(2):293-297.

(31) Alberti, Kurt George Matthew Mayer, Zimmet P. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. Diabetic Med 1998;15(7):539-553.

(32) Genuth S, Alberti KG, Bennett P, Buse J, Defronzo R, Kahn R, et al. Follow-up report on the diagnosis of diabetes mellitus. Diabetes Care 2003 Nov;26(11):3160-3167.

(33) Balkau B, Hu G, Qiao Q, Tuomilehto J, Borch-Johnsen K, Pyorala K, et al. Prediction of the risk of cardiovascular mortality using a score that includes glucose as a risk factor. The DECODE Study. Diabetologia 2004 Dec;47(12):2118-2128.

(34) World Health Organization. Use of glycated haemoglobin (HbA1c) in the diagnosis of diabetes mellitus. World Health Organization 2011.

(35) Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, Heine RJ. Translating the A1C assay into estimated average glucose values. Diabetes Care 2008;31(8):1473-1478.

(36) World Health Organization, World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. Geneva: World Health Organization 2006:1-50.

(37) Hjellestad ID, Astor MC, Nilsen RM, Søfteland E, Jonung T. HbA1c versus oral glucose tolerance test as a method to diagnose diabetes mellitus in vascular surgery patients. Cardiovascular diabetology 2013;12(1):79.

(38) Cavagnolli G, Comerlato J, Comerlato C, Renz PB, Gross JL, Camargo JL. HbA(1c) measurement for the diagnosis of diabetes: is it enough? Diabet Med 2011 Jan;28(1):31-35.

(39) Zemlin AE, Matsha TE, Hassan MS, Erasmus RT. HbA1c of 6.5% to Diagnose Diabetes Mellitus—Does It Work for Us?—The Bellville South Africa Study. PloS one 2011;6(8):e22558.

(40) Bonora E, Tuomilehto J. The pros and cons of diagnosing diabetes with A1C. Diabetes Care 2011 May;34(Supplement 2):S184-S190.

(41) Sacks DB. A1C versus glucose testing: a comparison. Diabetes Care 2011;34(2):518-523.

(42) Selvin E, Crainiceanu CM, Brancati FL, Coresh J. Short-term variability in measures of glycemia and implications for the classification of diabetes. Arch Intern Med 2007;167(14):1545.

(43) Park K, Saudek CD, Hart GW. Increased Expression of β-N-Acetylglucosaminidase in Erythrocytes From Individuals With Pre-diabetes and Diabetes. Diabetes 2010;59(7):1845-1850.

(44) De Vegt F, Dekker J, Ruhe H, Stehouwer C, Nijpels G, Bouter L, et al. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. Diabetologia 1999;42(8):926-931.

(45) Anderwald C, Gastaldelli A, Tura A, Krebs M, Promintzer-Schifferl M, Kautzky-Willer A, et al. Mechanism and effects of glucose absorption during an oral glucose tolerance test among females and males. Journal of Clinical Endocrinology & Metabolism 2011;96(2):515-524.

(46) Wolffenbuttel BH, Herman WH, Gross JL, Dharmalingam M, Jiang HH, Hardin DS. Ethnic Differences in Glycemic Markers in Patients With Type 2 Diabetes. Diabetes Care 2013.

(47) Sundaram R, Selvaraj N, Vijayan G, Bobby Z, Hamide A, Rattina Dasse N. Increased plasma malondialdehyde and fructosamine in iron deficiency anemia: effect of treatment. Biomedicine & Pharmacotherapy 2007;61(10):682-685.

(48) Polgreen PM, Putz D, Stapleton JT. Inaccurate Glycosylated Hemoglobin A1C Measurements in Human Immunodeficiency Virus—Positive Patients with Diabetes Mellitus. Clinical Infectious Diseases 2003;37(4):e53-e56.

(49) George JA. Should haemoglobin A 1c be used for the diagnosis of diabetes mellitus in South Africa? Journal of Endocrinology, Metabolism and Diabetes of South Africa 2011;16(3):122-129.

(50) Madeira FB, Silva AA, Veloso HF, Goldani MZ, Kac G, Cardoso VC, et al. Normal weight obesity is associated with metabolic syndrome and insulin resistance in young adults from a middle-income country. PLoS One 2013;8(3):e60673.

(51) Abate N, Chandalia M. Ethnicity and type 2 diabetes: focus on Asian Indians. J Diabetes Complications 2001 Nov-Dec;15(6):320-327.

(52) Thayer KA, Heindel JJ, Bucher JR, Gallo MA. Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. Environ Health Perspect 2012 Jun;120(6):779-789.

(53) Evans JM, Newton RW, Ruta DA, MacDonald TM, Morris AD. Socio-economic status, obesity and prevalence of Type 1 and Type 2 diabetes mellitus. Diabet Med 2000 Jun;17(6):478-480.

(54) Sanghera DK, Blackett PR. Type 2 Diabetes Genetics: Beyond GWAS. J Diabetes Metab 2012 Jun 23;3(198):6948.

(55) Lin Y, Sun Z. Current views on type 2 diabetes. J Endocrinol 2010;204(1):1-11.

(56) McKenney RL, Short DK. Tipping the balance: the pathophysiology of obesity and type 2 diabetes mellitus. Surg Clin North Am 2011;91(6):1139-1148.

(57) Rana JS, Li TY, Manson JE, Hu FB. Adiposity compared with physical inactivity and risk of type 2 diabetes in women. Diabetes Care 2007 Jan;30(1):53-58.

(58) Girgis CM, Gunton JE, Cheung NW. The influence of ethnicity on the development of type 2 diabetes mellitus in women with gestational diabetes: a prospective study and review of the literature. ISRN Endocrinol 2012 Apr 17;2012:341638.

(59) Hatzfeld JJ, LaVeist TA, Gaston-Johansson FG. Racial/ethnic disparities in the prevalence of selected chronic diseases among US Air Force members, 2008. Prev Chronic Dis 2012 Jun 14;9:112.

(60) Nazare JA, Smith JD, Borel AL, Haffner SM, Balkau B, Ross R, et al. Ethnic influences on the relations between abdominal subcutaneous and visceral adiposity, liver fat, and

cardiometabolic risk profile: the International Study of Prediction of Intra-Abdominal Adiposity and Its Relationship With Cardiometabolic Risk/Intra-Abdominal Adiposity. Am J Clin Nutr 2012 Oct;96(4):714-726.

(61) Gastaldelli A. Abdominal fat: does it predict the development of type 2 diabetes? Am J Clin Nutr 2008 May;87(5):1118-1119.

(62) Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. Diabetes 1998 May;47(5):699-713.

(63) Stringhini S, Batty GD, Bovet P, Shipley MJ, Marmot MG, Kumari M, et al. Association of lifecourse socioeconomic status with chronic inflammation and type 2 diabetes risk: the Whitehall II prospective cohort study. PLoS Med 2013 Jul;10(7):e1001479.

(64) Bonnefond A, Froguel P, Vaxillaire M. The emerging genetics of type 2 diabetes. Trends Mol Med 2010 Sep;16(9):407-416.

(65) Schäfer SA, Machicao F, Fritsche A, Häring H, Kantartzis K. New type 2 diabetes risk genes provide new insights in insulin secretion mechanisms. Diabetes Res Clin Pract 2011;93:S9-S24.

(66) Rewers M, Hamman RF. Risk factors for non-insulin-dependent diabetes. Diabetes in America 1995;2:179-220.

(67) Lehman DM, Fu D, Freeman AB, Hunt KJ, Leach RJ, Johnson-Pais T, et al. A Single Nucleotide Polymorphism in MGEA5 Encoding O-GlcNAc–selective N-Acetyl-β-d Glucosaminidase Is Associated With Type 2 Diabetes in Mexican Americans. Diabetes 2005;54(4):1214-1221.

(68) Steck AK, Winter WE. Review on monogenic diabetes. Curr Opin Endocrinol Diabetes Obes 2011 Aug;18(4):252-258.

(69) Lin Y, Sun Z. Current views on type 2 diabetes. J Endocrinol 2010;204(1):1-11.

(70) Collins FS, Morgan M, Patrinos A. The Human Genome Project: lessons from large-scale biology. Science 2003 Apr 11;300(5617):286-290.

(71) Feero WG, Guttmacher AE, McCarthy MI. Genomics, type 2 diabetes, and obesity. N Engl J Med 2010;363(24):2339-2350.

(72) Slomko H, Heo HJ, Einstein FH. Minireview: Epigenetics of obesity and diabetes in humans. Endocrinology 2012 Mar;153(3):1025-1030.

(73) Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A Common Variant in the FTO Gene Is Associated with Body Mass Index and Predisposes to Childhood and Adult Obesity. Science 2007 May 11;316(5826):889-894.

(74) Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. Cell 2012 Mar 2;148(5):852-871.

(75) McMurray F, Cox RD. Mouse models and type 2 diabetes: translational opportunities. Mammalian Genome 2011;22(7-8):390-400.

(76) The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus, Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, et al. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. Journal of Diabetes Investigation 2010;1(5):212-228.

(77) Muoio DM, Newgard CB. Molecular and metabolic mechanisms of insulin resistance and β-cell failure in type 2 diabetes. Nature reviews Molecular cell biology 2008;9(3):193-205.

(78) Shulman GI. Cellular mechanisms of insulin resistance. J Clin Invest 2000;106(2):171-176.

(79) Rolo AP, Palmeira CM. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol 2006 Apr 15;212(2):167-178.

(80) Saini V. Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. World J Diabetes 2010 Jul 15;1(3):68-75.

(81) Bouché C, Serdy S, Kahn CR, Goldfine AB. The cellular fate of glucose and its relevance in type 2 diabetes. Endocr Rev 2004;25(5):807-830.

(82) Choi K, Kim Y. Molecular mechanism of insulin resistance in obesity and type 2 diabetes. Korean J Intern Med 2010;25(2):119-129.

(83) Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, et al. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proceedings of the National Academy of Sciences 2001;98(8):4640-4645.

(84) Nakashima N, Sharma PM, Imamura T, Bookstein R, Olefsky JM. The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. J Biol Chem 2000;275(17):12889-12895.

(85) Bourdeau A, Dube N, Tremblay ML. Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol 2005 Apr;17(2):203-209.

(86) Gual P, Le Marchand-Brustel Y, Tanti J. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 2005 1;87(1):99-109.

(87) Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007;129(7):1261-1274.

(88) Barthel A, Schmoll D. Novel concepts in insulin regulation of hepatic gluconeogenesis. American Journal of Physiology-Endocrinology And Metabolism 2003;285(4):E685-E692.

(89) Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, et al. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. American Journal of Physiology-Endocrinology And Metabolism 2003;285(4):E718-E728.

(90) Jaworski K, Sarkadi-Nagy E, Duncan RE, Ahmadian M, Sul HS. Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue. American Journal of Physiology-Gastrointestinal and Liver Physiology 2007;293(1):G1-G4.

(91) Carmen G, Víctor S. Signalling mechanisms regulating lipolysis. Cell Signal 2006;18(4):401-408.

(92) Reaven GM, Hollenbeck C, Jeng C, Wu MS. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. Diabetes 1988;37(8):1020-1024.

(93) Paolisso G, Tataranni P, Foley J, Bogardus C, Howard B. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. Diabetologia 1995;38(10):1213-1217.

(94) Randle P, Garland P, Hales C, Newsholme E. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. The Lancet 1963;281(7285):785-789.

(95) Randle P, Garland P, Newsholmet E, Hales C. The glucose fatty acid cycle in obesity and maturity onset diabetes mellitus. Ann N Y Acad Sci 1965;131(1):324-333.

(96) Qatanani M, Lazar MA. Mechanisms of obesity-associated insulin resistance: many choices on the menu. Genes Dev 2007;21(12):1443-1455.

(97) Poornima IG, Parikh P, Shannon RP. Diabetic Cardiomyopathy The Search for a Unifying Hypothesis. Circ Res 2006;98(5):596-605.

(98) Polonsky K, Given B, Van Cauter E. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. J Clin Invest 1988;81(2):442.

(99) Dankner R, Chetrit A, Shanik MH, Raz I, Roth J. Basal-State Hyperinsulinemia in Healthy Normoglycemic Adults Is Predictive of Type 2 Diabetes Over a 24-Year Follow-Up A preliminary report. Diabetes Care 2009;32(8):1464-1466.

(100) Weyer C, Bogardus C, Mott DM, Pratley RE. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest 1999;104(6):787-794.

(101) Lebovitz HE. Insulin: potential negative consequences of early routine use in patients with type 2 diabetes. Diabetes Care 2011 May;34(Supplement 2):S225-S230.

(102) Rhodes CJ. Type 2 diabetes-a matter of beta-cell life and death? Science 2005 Jan 21;307(5708):380-384.

(103) Ramlo-Halsted BA, Edelman SV. The natural history of type 2 diabetes: practical points to consider in developing prevention and treatment strategies. Clinical Diabetes 2000;18(2):80-84.

(104) Bjornholm M, Zierath JR. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. Biochem Soc Trans 2005 Apr;33(Pt 2):354-357.

(105) Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001 Dec 13;414(6865):813-820.

(106) Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005 Jun;54(6):1615-1625.

(107) Fernández-Mejía C. Oxidative Stress in Diabetes Mellitus and the Role Of Vitamins with Antioxidant Actions. OXIDATIVE STRESS AND CHRONIC DEGENERATIVE DISEASES-A ROLE FOR ANTIOXIDANTS 2013:209.

(108) Kuka S, Tatarkova Z, Kaplan P. Oxidative Damage to Proteins and Lipids During Ageing. Acta Medica Martiniana 2012 Sep;12(1):5-11.

(109) Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res 2010 Oct 29;107(9):1058-1070.

(110) Siebels I, Dröse S. Q-site inhibitor induced ROS production of mitochondrial complex II is attenuated by TCA cycle dicarboxylates. Biochimica et Biophysica Acta (BBA) - Bioenergetics 2013 Oct;1827(10):1156-1164.

(111) Trumpower BL. The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. J Biol Chem 1990 Jul 15;265(20):11409-11412.

(112) Mohora M, Greabu M, Muscurel C, Duţă C, Totan A. The sources and the targets of oxidative stress in the etiology of diabetic complications. Romanian J Biophys 2007;17:63-84.

(113) Orasanu G, Plutzky J. The pathologic continuum of diabetic vascular disease. J Am Coll Cardiol 2009;53(5s1):S35-S42.

(114) Henriksen EJ, Diamond-Stanic MK, Marchionne EM. Oxidative stress and the etiology of insulin resistance and type 2 diabetes. Free Radical Biology and Medicine 2011;51(5):993-999.

(115) Pacher P, Szabó C. Role of poly (ADP-ribose) polymerase-1 activation in the pathogenesis of diabetic complications: endothelial dysfunction, as a common underlying theme. Antioxidants & redox signaling 2005;7(11-12):1568-1580.

(116) Lorenzi M. The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. Journal of Diabetes Research 2007 Jul 30;2007:61038.

(117) Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev 2008;88(4):1341-1378.

(118) Geraldes P, King GL. Activation of protein kinase C isoforms and its impact on diabetic complications. Circ Res 2010;106(8):1319-1331.

(119) Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL. Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. Diabetes 1994;43(9):1122-1129.

(120) Scivittaro V, Ganz MB, Weiss MF. AGEs induce oxidative stress and activate protein kinase C-βII in neonatal mesangial cells. American Journal of Physiology-Renal Physiology 2000;278(4):F676-F683.

(121) Keogh RJ, Dunlop ME, Larkins RG. Effect of inhibition of aldose reductase on glucose flux, diacylglycerol formation, protein kinase C, and phospholipase A2 activation. Metab Clin Exp 1997;46(1):41-47.

(122) Koya D, Jirousek MR, Lin Y, Ishii H, Kuboki K, King GL. Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. J Clin Invest 1997;100(1):115.

(123) Studer RK, Craven PA, DeRubertis FR. Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. Diabetes 1993;42(1):118-126.

(124) Way K, Katai N, King G. Protein kinase C and the development of diabetic vascular complications. Diabetic Med 2001;18(12):945-959.

(125) Degenhardt T, Thorpe S, Baynes J. Chemical modification of proteins by methylglyoxal. Cell Mol Biol 1998;44(7):1139-1145.

(126) Yao D, Brownlee M. Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. Diabetes 2010;59(1):249-255.

(127) Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovasc Res 1998;37(3):586-600.

(128) Marshall S, Bacote V, Traxinger R. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem 1991;266(8):4706-4712.

(129) Buse MG. Hexosamines, insulin resistance, and the complications of diabetes: current status. American Journal of Physiology-Endocrinology And Metabolism 2006;290(1):E1-E8.

(130) Wells L, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ, Hart GW. Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. Molecular & Cellular Proteomics 2002;1(10):791-804.

(131) Teo CF, Wollaston-Hayden EE, Wells L. Hexosamine flux, the O-GlcNAc modification, and the development of insulin resistance in adipocytes. Mol Cell Endocrinol 2010;318(1):44-53.

(132) Wamelink M, Struys E, Jakobs C. The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. J Inherit Metab Dis 2008;31(6):703-717.

(133) Zhao J, Zhong C. A review on research progress of transketolase. Neuroscience bulletin 2009;25(2):94-99.

(134) Hammes H, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, et al. Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. Nat Med 2003;9(3):294-299.

(135) Katare R, Oikawa A, Cesselli D, Beltrami AP, Avolio E, Muthukrishnan D, et al. Boosting the pentose phosphate pathway restores cardiac progenitor cell availability in diabetes. Cardiovasc Res 2013;97(1):55-65.

(136) Katare RG, Caporali A, Oikawa A, Meloni M, Emanueli C, Madeddu P. Vitamin B1 Analog Benfotiamine Prevents Diabetes-Induced Diastolic Dysfunction and Heart Failure Through Akt/Pim-1–Mediated Survival Pathway. Circulation: Heart Failure 2010;3(2):294-305.

(137) Haupt E, Ledermann H, Köpcke W. Benfotiamine in the treatment of diabetic polyneuropathy: a three-week randomized, controlled pilot study (BEDIP study). Int J Clin Pharmacol Ther 2005;43(2):71-77.

(138) Serpillon S, Floyd BC, Gupte RS, George S, Kozicky M, Neito V, et al. Superoxide production by NAD (P) H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac dysfunction. The role of glucose-6-phosphate dehydrogenase-derived NADPH. American Journal of Physiology-Heart and Circulatory Physiology 2009;297(1):H153-H162.

(139) Saito N, Kimura M, Kuchiba A, Itokawa Y. The relationship between blood thiamine levels and dietary thiamine content in diabetic outpatients and healthy subjects. J Nutr Sci Vitaminol (Tokyo) 1987 Dec;33(6):431-438.

(140) Glinsky GV, Krones-Herzig A, Glinskii AB. Malignancy-associated regions of transcriptional activation: gene expression profiling identifies common chromosomal regions of a recurrent transcriptional activation in human prostate, breast, ovarian, and colon cancers. Neoplasia (New York, NY) 2003;5(3):218.

(141) Coy JF, Dressler D, Wilde J, Schubert P. Mutations in the transketolase-like gene TKTL1: clinical implications for neurodegenerative diseases, diabetes and cancer. Clin Lab 2005;51(5-6):257-274.

(142) Coy JF, Dübel S, Kioschis P, Thomas K, Micklem G, Delius H, et al. Molecular cloning of tissue-specific transcripts of a transketolase-related gene: implications for the evolution of new vertebrate genes. Genomics 1996;32(3):309-316.

(143) Schneider S, Lüdtke S, Schröder-Tittmann K, Wechsler C, Meyer D, Tittmann K. A Δ38 Deletion Variant of Human Transketolase as a Model of Transketolase-Like Protein 1 Exhibits No Enzymatic Activity. PloS one 2012;7(10):e48321.

(144) Chen H, Yue J, Yang S, Ding H, Zhao R, Zhang S. Overexpression of transketolase-like gene 1 is associated with cell proliferation in uterine cervix cancer. Journal of Experimental & Clinical Cancer Research 2009;28(1):43.

(145) Feyen O, Coy JF, Prasad V, Schierl R, Saenger J, Baum RP. EDIM-TKTL1 blood test: a noninvasive method to detect upregulated glucose metabolism in patients with malignancies. Future Oncology 2012;8(10):1349-1359.

(146) Bond MR, Hanover JA. O-GlcNAc Cycling: A Link Between Metabolism and Chronic Disease. Annu Rev Nutr 2013 Apr 29;33:205-229.

(147) Torres C, Hart GW. Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. J Biol Chem 1984;259(5):3308-3317.

(148) Ngoh GA, Facundo HT, Zafir A, Jones SP. O-GlcNAc signaling in the cardiovascular system. Circ Res 2010;107(2):171-185.

(149) Hart GW, Housley MP, Slawson C. Cycling of O-linked β-N-acetylglucosamine on nucleocytoplasmic proteins. Nature 2007;446(7139):1017-1022.

(150) Zachara NE, O'Donnell N, Cheung WD, Mercer JJ, Marth JD, Hart GW. Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress A survival response of mammalian cells. J Biol Chem 2004;279(29):30133-30142.

(151) Marshall S, Bacote V, Traxinger R. Complete inhibition of glucose-induced desensitization of the glucose transport system by inhibitors of mRNA synthesis. Evidence for rapid turnover of glutamine: fructose-6-phosphate amidotransferase. J Biol Chem 1991;266(16):10155-10161.

(152) Ruan H, Singh JP, Li M, Wu J, Yang X. Cracking the< i> O</i>-GlcNAc code in metabolism. Trends in Endocrinology & Metabolism 2013 Jun;24(6):301-309.

(153) Cooksey RC, McCLAIN DA. Transgenic Mice Overexpressing the Rate-Limiting Enzyme for Hexosamine Synthesis in Skeletal Muscle or Adipose Tissue Exhibit Total Body Insulin Resistance. Ann N Y Acad Sci 2002;967(1):102-111.

(154) Hazel M, Cooksey RC, Jones D, Parker G, Neidigh JL, Witherbee B, et al. Activation of the hexosamine signaling pathway in adipose tissue results in decreased serum adiponectin and skeletal muscle insulin resistance. Endocrinology 2004;145(5):2118-2128.

(155) Hebert Jr LF, Daniels MC, Zhou J, Crook ED, Turner RL, Simmons ST, et al. Overexpression of glutamine: fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. J Clin Invest 1996;98(4):930.

(156) Vosseller K, Wells L, Lane MD, Hart GW. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. Proceedings of the National Academy of Sciences 2002;99(8):5313-5318.

(157) Robinson KA, Ball LE, Buse MG. Reduction of O-GlcNAc protein modification does not prevent insulin resistance in 3T3-L1 adipocytes. American Journal of Physiology-Endocrinology And Metabolism 2007;292(3):E884-E890.

(158) Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, et al. Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. Nature 2008;451(7181):964-969.

(159) Springhorn C, Matsha TE, Erasmus RT, Essop MF. Exploring Leukocyte O-GlcNAcylation as a Novel Diagnostic Tool for the Earlier Detection of Type 2 Diabetes Mellitus. Journal of Clinical Endocrinology & Metabolism 2012;97(12):4640-4649.

(160) Butkinaree C, Park K, Hart GW. O-linked β-< i> N</i>-acetylglucosamine (< i> O</i>-GlcNAc): Extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. Biochimica et Biophysica Acta (BBA)-General Subjects 2010;1800(2):96-106.

(161) Wang Z, Gucek M, Hart GW. Cross-talk between GlcNAcylation and phosphorylation: site-specific phosphorylation dynamics in response to globally elevated O-GlcNAc. Proceedings of the National Academy of Sciences 2008;105(37):13793-13798.

(162) Hurtado-Guerrero R, Dorfmueller HC, Van Aalten DM. Molecular mechanisms of< i> O</i>-GlcNAcylation. Curr Opin Struct Biol 2008;18(5):551-557.

(163) Zachara NE, Hart GW. < i> O</i>-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. Biochimica et Biophysica Acta (BBA)-General Subjects 2004;1673(1):13-28.

(164) Park SY, Ryu J, Lee W. O-GlcNAc modification on IRS-1 and Akt2 by PUGNAc inhibits their phosphorylation and induces insulin resistance in rat primary adipocytes. Exp Mol Med 2005;37(3):220-229.

(165) Gu Y, Ande SR, Mishra S. Altered< i> O</i>-GlcNAc modification and phosphorylation of mitochondrial proteins in myoblast cells exposed to high glucose. Arch Biochem Biophys 2011;505(1):98-104.

(166) Liu J, Jahn LA, Fowler DE, Barrett EJ, Cao W, Liu Z. Free fatty acids induce insulin resistance in both cardiac and skeletal muscle microvasculature in humans. Journal of Clinical Endocrinology & Metabolism 2011;96(2):438-446.

(167) Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, Hunt DF, et al. O-GlcNAc regulates FoxO activation in response to glucose. J Biol Chem 2008;283(24):16283-16292.

(168) Vocadlo DJ. O-GlcNAc processing enzymes: catalytic mechanisms, substrate specificity, and enzyme regulation. Curr Opin Chem Biol 2012 Dec;16(5-6):488-497.

(169) Kelly WG, Dahmus ME, Hart GW. RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. J Biol Chem 1993;268(14):10416-10424.

(170) Kim EJ. Chemical arsenal for the study of O-GlcNAc. Molecules 2011;16(3):1987-2022.

(171) Nolte D, Müller U. Human O-GlcNAc transferase (OGT): genomic structure, analysis of splice variants, fine mapping in Xq13. 1. Mammalian genome 2002;13(1):62-64.

(172) O'Donnell N, Zachara NE, Hart GW, Marth JD. Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. Mol Cell Biol 2004;24(4):1680-1690.

(173) Lubas WA, Hanover JA. Functional expression of O-linked GlcNAc transferase Domain structure and substrate specificity. J Biol Chem 2000;275(15):10983-10988.

(174) Kreppel LK, Blomberg MA, Hart GW. Dynamic glycosylation of nuclear and cytosolic proteins cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J Biol Chem 1997;272(14):9308-9315.

(175) Hanover JA, Yu S, Lubas WB, Shin S, Ragano-Caracciola M, Kochran J, et al. Mitochondrial and nucleocytoplasmic isoforms of O-linked GlcNAc transferase encoded by a single mammalian gene. Arch Biochem Biophys 2003;409(2):287-297.

(176) Kreppel LK, Blomberg MA, Hart GW. Dynamic glycosylation of nuclear and cytosolic proteins cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J Biol Chem 1997;272(14):9308-9315.

(177) Issad T, Masson E, Pagesy P. O-GlcNAc modification, insulin signaling and diabetic complications. Diabetes Metab 2010;36(6):423-435.

(178) Dong D, Hart GW. Purification and characterization of an O-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J Biol Chem 1994;269(30):19321-19330.

(179) Kim EJ, Kang DO, Love DC, Hanover JA. Enzymatic characterization of< i> O</i>-GlcNAcase isoforms using a fluorogenic GlcNAc substrate. Carbohydr Res 2006;341(8):971-982.

(180) DeHaven JE, Robinson KA, Nelson BA, Buse MG. A Novel Variant of Glutamine Fructose-6-Phosphate Amidotransferase-1 (GFAT1) mRNA Is Selectively Expressed in Striated Muscle. Diabetes 2001;50(11):2419-2424.

(181) Whitmore T, Mudri S, McKnight G. Physical mapping of the human glutamine: fructose-6-phosphate amidotransferase gene (GFPT) to chromosome 2p13. Genomics 1995;26(2):422-423.

(182) Oki T, Yamazaki K, Kuromitsu J, Okada M, Tanaka I. cDNA cloning and mapping of a novel subtype of glutamine: fructose-6-phosphate amidotransferase (GFAT2) in human and mouse. Genomics 1999;57(2):227-234.

(183) Laczy B, Hill BG, Wang K, Paterson AJ, White CR, Xing D, et al. Protein O-GlcNAcylation: a new signaling paradigm for the cardiovascular system. American Journal of Physiology-Heart and Circulatory Physiology 2009;296(1):H13-H28.

(184) Srinivasan V, Sandhya N, Sampathkumar R, Farooq S, Mohan V, Balasubramanyam M. Glutamine fructose-6-phosphate amidotransferase (GFAT) gene expression and activity in patients with type 2 diabetes: inter-relationships with hyperglycaemia and oxidative stress. Clin Biochem 2007;40(13):952-957.

(185) Yki-Järvinen H, Daniels MC, Virkamäki A, Mäkimattila S, DeFronzo RA, McClain D. Increased glutamine: fructose-6-phosphate amidotransferase activity in skeletal muscle of patients with NIDDM. Diabetes 1996;45(3):302-307.

(186) Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611-622.

(187) Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR. Basic principles of real-time quantitative PCR. Expert review of molecular diagnostics 2005;5(2):209-219.

(188) Maeß M, Sendelbach S, Lorkowski S. Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. BMC molecular biology 2010;11(1):90.

(189) Springhorn C. Leukocyte O-GlcNAcylation: a novel diagnostic tool for the earlier detection of type 2 diabetes mellitus? 2012.

(190) Christensen DL, Witte DR, Kaduka L, Jørgensen ME, Borch-Johnsen K, Mohan V, et al. Moving to an A1C-based diagnosis of diabetes has a different impact on prevalence in different ethnic groups. Diabetes Care 2010;33(3):580-582.

(191) Burt D, Brodbeck K, Häring HU, Schleicher ED, Weigert C. Partial characterisation of the human GFAT promoter: Effect of single nucleotide polymorphisms on promoter function. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 2005;1740(1):85-90.

(192) Robinson KA, Weinstein ML, Lindenmayer GE, Buse MG. Effects of diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. Diabetes 1995;44(12):1438-1446.

(193) Chang Q, Su K, Baker JR, Yang X, Paterson AJ, Kudlow JE. Phosphorylation of human glutamine: fructose-6-phosphate amidotransferase by cAMP-dependent protein kinase at serine 205 blocks the enzyme activity. J Biol Chem 2000;275(29):21981-21987.

(194) Zhang H, Jia Y, Cooper JJ, Hale T, Zhang Z, Elbein SC. Common variants in glutamine: fructose-6-phosphate amidotransferase 2 (GFPT2) gene are associated with type 2 diabetes, diabetic nephropathy, and increased GFPT2 mRNA levels. Journal of Clinical Endocrinology & Metabolism 2004;89(2):748-755.

(195) Erasmus RT, Soita DJ, Hassan MS, Blanco-Blanco E, Vergotine Z, Kengne AP, et al. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. South African Medical Journal 2012;102(11):841-844.

(196) Waheed P, Naveed AK, Ahmed T. Thiamine deficiency and its correlation with dyslipidaemia in diabetics with microalbuminuria. JPMA 2013;63(340).

(197) Frank RA, Titman CM, Pratap JV, Luisi BF, Perham RN. A molecular switch and proton wire synchronize the active sites in thiamine enzymes. Science 2004;306(5697):872-876.

(198) Meshalkina LE, Drutsa VL, Koroleva ON, Solovjeva ON, Kochetov GA. Is transketolase-like protein, TKTL1, transketolase? Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 2012; 1832(3):387-390.

(199) Hu L, Yang J, Zhang D, Zhang S, Wang L, Cai P, et al. The TKTL1 gene influences total transketolase activity and cell proliferation in human colon cancer LoVo cells. Anticancer Drugs 2007;18(4):427-433.

(200) Wittig R, Coy JF. The role of glucose metabolism and glucose-associated signalling in cancer. Perspectives in medicinal chemistry 2007;1:64.

(201) Chen H, Yue J, Yang S, Ding H, Zhao R, Zhang S. Overexpression of transketolase-like gene 1 is associated with cell proliferation in uterine cervix cancer. Journal of Experimental & Clinical Cancer Research 2009;28(1):43.

(202) Schwaab J, Horisberger K, Ströbel P, Bohn B, Gencer D, Kähler G, et al. Expression of Transketolase like gene 1 (TKTL1) predicts disease-free survival in patients with locally advanced rectal cancer receiving neoadjuvant chemoradiotherapy. BMC Cancer 2011;11(1):363.

(203) Prasad P, Tiwari AK, Kumar KP, Ammini A, Gupta A, Gupta R, et al. Association analysis of ADPRT1, AKR1B1, RAGE, GFPT2 and PAI-1 gene polymorphisms with chronic renal insufficiency among Asian Indians with type-2 diabetes. BMC medical genetics 2010;11(1):52.

(204) Nishimura C, Saito T, Ito T, Omori Y, Tanimoto T. High levels of erythrocyte aldose reductase and diabetic retinopathy in NIDDM patients. Diabetologia 1994;37(3):328-330.

(205) Hasegawa G, Obayashi H, Kitamura A, Hashimoto M, Shigeta H, Nakamura N, et al. Increased levels of aldose reductase in peripheral mononuclear cells from type 2 diabetic patients with microangiopathy. Diabetes Res Clin Pract 1999;45(1):9-14.

(206) Shimizu H, Ohtani K, Tsuchiya T, Sato N, Tanaka Y, Takahashi H, et al. Aldose reductase mRNA expression is associated with rapid development of diabetic microangiopathy in Japanese Type 2 diabetic (T2DM) patients. Diabetes Nutr Metab 2000;13(2):75-79.

(207) Hanover JA, Krause MW, Love DC. Bittersweet memories: linking metabolism to epigenetics through O-GlcNAcylation. Nature Reviews Molecular Cell Biology 2012;13(5):312-321.

(208) Molenkamp R, van der Ham A, Schinkel J, Beld M. Real-Time Multiplex PCR of five Different DNA Targets Using the LightCycler® 480 System. BIOCHEMICA-MANNHEIM-2007;3:15.

(209) Rutledge RG, Stewart D. Assessing the performance capabilities of LRE-based assays for absolute quantitative real-time PCR. PloS one 2010;5(3):e9731.