Investigating changes to the insulin signalling pathway in a diet-induced pre-diabetic rat model: Effects on selected markers

By

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Discipline of Human Physiology

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Declaration

I, Aubrey Mbulelo Sosibo hereby declare that the dissertation entitled:

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is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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Plagiarism declaration

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List of abbreviations

ADA	American Diabetes Association
ATP	Adenosine triphosphate
AGEs	Advanced glycosylated end products
Akt/PKB	Protein Kinase beta
AUC	Area Under Curve
CAP	Cbl-associated protein
Cbl	Casitas b-lineage lymphoma
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
4E-BP	4E-binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
GBE	Glycogen branching enzyme
GSK3	Glycogen synthase kinase 3
HbA1c	Glycated haemoglobin

HRP	Horse radish peroxidase
HFHC-D	High fat high carbohydrates Diet
HOMA	Homeostasis Model Assessment
IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose
IRS	Insulin receptor substrate
IR	Insulin resistance
IL-6	Interleukin 6
JNK	c-JUN N-terminal kinase
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
ND	Normal diet
OGT	Oral glucose tolerance
PKC	Protein kinase C
KOH	Potassium hydroxide
PI3K	Phosphoinositol 3 kinase
p70S6K	Protein S6 kinase

PCR	Polymerase chain reaction
PDK	Phosphoinositide-dependant protein kinase-1
PIP2	Phosphatidylinositol (4, 5)-bisphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
RNA	Ribonucleic acid
SEM	Standard error of means
T2DM	Type 2 diabetes mellitus
TNF	Tumor necrosis factor
UKZN	University of KwaZulu-Natal
UDP	Uridine 5′-diphosphate
WHO	World Health Organization

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Chapter 1: Literature review

1.0 Background

Diabetes mellitus is defined as a polygenic [1] and a long-lasting metabolic disease that disturbs the body's ability to utilize glucose molecules derived from broken down carbohydrates [2]. The body's inability to utilize glucose is due to pancreatic beta (β)-cell functional impairment and/or insulin resistance (IR) in insulin-dependent tissues such as skeletal muscle [3, 4]. Consequently, blood glucose levels rise resulting in hyperglycaemia [5]. Under normal conditions, the body will without any restriction be able to passively and actively take in glucose molecules into cells where they will serve as fuel required by the body [6]. However, for active glucose transport, the cells such as those found in the adipose, cardiac and skeletal muscle tissue require insulin to be secreted into the bloodstream in order to utilize glucose from the blood [7]. Therefore, when these cells are under diabetic conditions where either the body fails to produce enough insulin, or the body is unable to use the insulin it produces or a combination of both [6]. This causes detrimental effects in the body and may result in type 2 diabetes mellitus (T2DM) that often lead to fatality.

Diabetes is a prevalent non-communicable disease that is regarded as one of the foremost public health concerns worldwide [6]. In a study conducted by Wild and Associates, they estimate a possible doubling of diabetes prevalence between the year 2000 and 2030 [8]. The World Health Organization (WHO) also reported that in the year 2014, a global estimate of 422 million of the adults from the age of eighteen upwards had diabetes, and in the year 2012 alone diabetes was directly responsible for 1.5 million deaths [9]. Interestingly, diabetes-related deaths occur mainly in low and middle-income countries and South Africa is one of the middle-income countries had

a 9.8% total prevalence of diabetes of its entire population [10, 11]. T2DM is the most prevalent of all types of diabetes and, it is estimated that T2DM prevalence will rise to 439 million by 2030 [2]. These statistics are alarming and are an indication of the ineffectiveness of current diabetic therapies and that a more potent intervention is needed to halt the rising prevalence and high mortality of diabetes mellitus.

The general attribute for T2DM is a hyperglycaemic condition that is preceded by IR [4], and T2DM is associated with lifestyle risks factors such as living a sedentary lifestyle and/or the consumption of an unhealthy diet [12]. A Western-style diet is rendered unhealthy because it incorporates excessive amounts of carbohydrates and fats in the form of refined grains, saturated fats and high-sweetened refined sugars [13]. Studies have shown that IR and T2DM are associated with diets rich in carbohydrates or saturated fats [14, 15]. Thus, Western-style diets are recognized as one of the major culprits for the development of diabetes [16]. Furthermore, these risk factors have been implicated in changes in expression of genes associated with the insulin signalling pathway [17]. It has been shown that in T2DM rats, the gene expression of proteins influencing the insulin signalling pathway is significantly altered [18]. Interestingly, findings in the literature show that the onset of chronic complications may well occur at least 4–7 years before clinical diagnosis of diabetes, which would simply mean that chronic complications occur prior to hyperglycaemia [19, 20]. Therefore, this suggests that T2DM may be diagnosed too late. Thus the particular interest in looking at a previously overlooked long-lasting condition that precedes T2DM, known as pre-diabetes [21, 22].

Pre-diabetes is now recognized as one of the frontline risk factors that leads to an overt T2DM [22]. The WHO describes pre-diabetes as a condition of intermediate hyperglycaemia, which is preceded by moderate IR [22]. Globally it is anticipated that more than 470 million people will

suffer from pre-diabetes by the year 2030 [22]. It is determined by using specific parameters such as impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and the glycated haemoglobin (HbA1c) [23]. A study conducted in our laboratory showed that the animals fed with high-fats high-carbohydrates diet (HFHC-D) developed pre-diabetes as shown by augmented glycosylated haemoglobin and moderate IR [24-26]. However, the mechanics leading to moderate IR have not yet been studied in this model. Thus, this study sought to investigate whether there are any changes in the expression of genes coded for proteins involved in the insulin signalling pathway during pre-diabetes using a diet-induced pre-diabetic rat model characterized with moderate impairment of the insulin sensitivity.

1.1 Insulin

Insulin is a hormone produced by pancreatic beta-cells in response to rising glucose levels, and its structure consists of two chains of amino acids connected by disulphide bridges [27]. The two chains of amino acids are differentiated as the A chains that compile 21 amino acids, and B chains that compile 30 amino acids [28]. This composition of 51 amino acids makes up the 5808 Da molecular weight of the protein insulin [28, 29]. Insulin is known to have multiple functions in the body as depicted in figure 1.

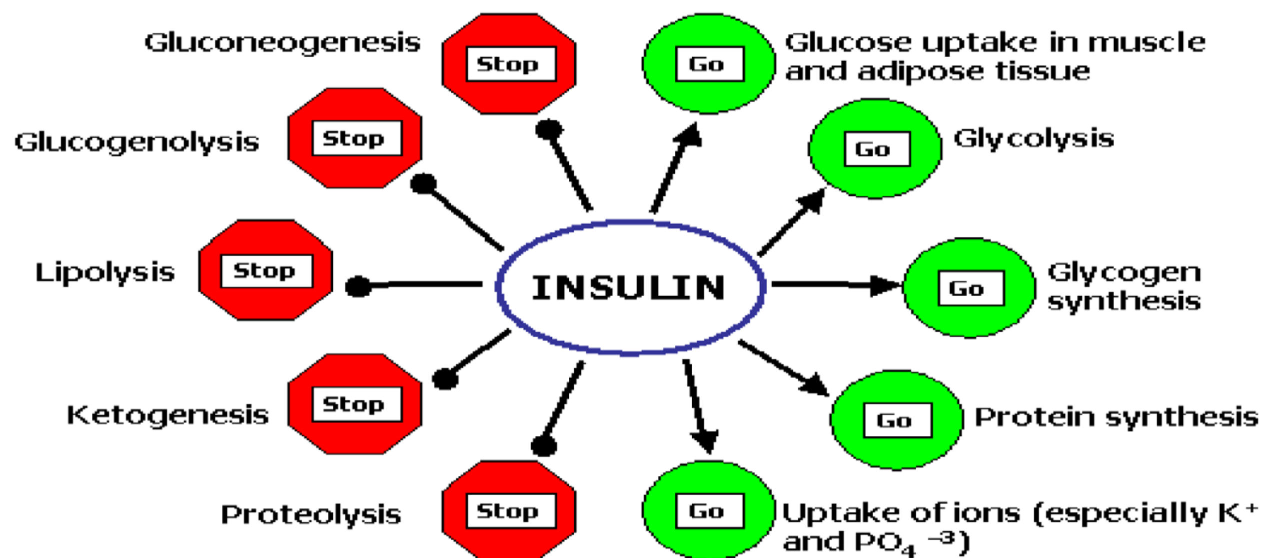

Figure 1: Actions of insulin (Martin, 1983). The above diagram displays the multiple functions of the hormone insulin, and its profound ability to determine the fate of energy-carrying molecules such as glucose, fats and lipids. With some undergoing oxidation and others being stored. However, for insulin to perform its multiple functions, it will require a functional insulin signalling pathway.

In order to carry out its functions, the hormone insulin binds to insulin receptors of the target cell that then trigger signal transduction [7]. Subsequently, insulin stimulates the cell to translocate the glucose transporter type 4 (GLUT-4) protein into its membrane. This allows the influx of glucose into the cell and fall in blood glucose levels, which then inhibits the pancreatic β-cells from releasing more insulin through a negative feedback mechanism [30]. Insulin is also regarded as an anabolic hormone and consequently elevates the storage of macromolecules like amino acids, glucose and fatty acids [31]. Consequently, the effects of insulin promote body weight gain [29]. In the absence of insulin, glucose uptake decreases hence increasing mobilisation of lipids in adipocytes [29, 32]. Inversely, in the presence of insulin, glucose uptake increases in the tissues

and decreases mobilization of lipids in adipocytes [29]. However, when in excess, insulin causes hypoglycaemia leading to convulsions and/or coma [32]. Another profound function of insulin is its ability to organize the use of fuels/energy carrying substances for either storage or oxidation [7]. Thus, insulin is able to inhibit gluconeogenesis and glycogenolysis, stimulates glycolysis and glycogenesis, stimulates uptake and incorporation of amino acids into protein, inhibits protein degradation, stimulates lipogenesis, and suppress lipolysis [28]. For the hormone insulin to perform its multiple functions, it requires a fully functional insulin signalling pathway.

1.2 Insulin signalling pathway

The insulin action is facilitated through insulin receptors, which propagate its activity by means of three different pathways: mitogen-activated protein kinase (MAPK) pathway, Cbl-associated protein (CAP) pathway and phosphatidylinositol-3 kinase (PI3K) pathway [12, 33]. such as

1.2.1 Mitogen-activated protein kinase (MAPK) pathway

The Mitogen-activated protein kinases (MAPK) are protein kinases that undergo autophosphorylation of their own dual serine and threonine residues or phosphorylate those found on their substrates to activate or de-activate their target [33]. Mitogen-activated protein kinases (MAPKs) are ubiquitously expressed and are responsible for regulating critical cellular processes such as proliferation, stress responses, apoptosis and immune defence [34]. The activation of a MAPK cascade come about in a segment of consecutive phosphorylation's. A MAPK segment comprises a MAP3K that activates a MAP2K, which then activate a MAPK [33, 35]. Mitogen-activated protein kinases (MAPK) protein phosphatases (MKPs) can inactivate MAPK phosphorylation events by dephosphorylating phosphothreonine and phosphotyrosine residues on

MAPKs [36]. In mammalian cells there exist three distinguished MAPK pathways: the c-JUN N-terminal kinase 1, 2 and 3 (JNK1/2/3), the ERK1/2, and the p38 MAPK α, β, δ, and γ pathways [37]. ERK1/2 is activated in response to growth factors, hormones and proinflammatory stimuli, while JNK 1/2/3 and p38 MAPK α, β, δ, and γ are activated by cellular and environmental stresses, in addition to proinflammatory stimuli [37, 38]. The inflammatory signals are known to cause IR by c-Jun N-terminal kinase-mediated phosphorylation of the IRS proteins. As a result, glucose homeostasis can become impaired [39].

1.2.2 Cbl-associated protein (CAP) pathway

Literature has shown that two pathways are necessary for insulin-stimulated glucose transport; a PI(3)K-dependent pathway and a PI(3)K-independent pathway [40]. However, recent data suggest that proto-oncogene, Casitas b-lineage lymphoma (Cbl), play a role in insulin action, independently of the PI(3)K/Akt pathway [41]. In an adipocyte cell line, 3T3-L1, the Cbl forms a compound with 2 adaptor proteins, an adaptor protein with Pleckstrin homology and Src homology 2 domains (APS) and CAP [42]. Then when induced by insulin, this complex binds to the insulin receptor via an interaction between an SH2 domain in APS and the tyrosyl-phosphorylated receptor tail [42]. Subsequently, insulin-dependent tyrosine phosphorylation occurs in Cbl, which in turn causes the recruitment of the CAP/Cbl complex to lipid rafts via an interaction between the CAP and the lipid raft protein flotillin [42, 43]. Thereafter, tyrosyl-phosphorylated Cbl recruits the CrkII/C3G heterodimer responsible for the activation of the small G protein TC10. The stimulation of TC10 delivers another signal to the glucose transporter 4 (GLUT-4) to undergo translocation [42, 44]. Therefore, the TC10 pathway may function in parallel with PI(3)K to stimulate fully GLUT4 translocation in response to insulin.

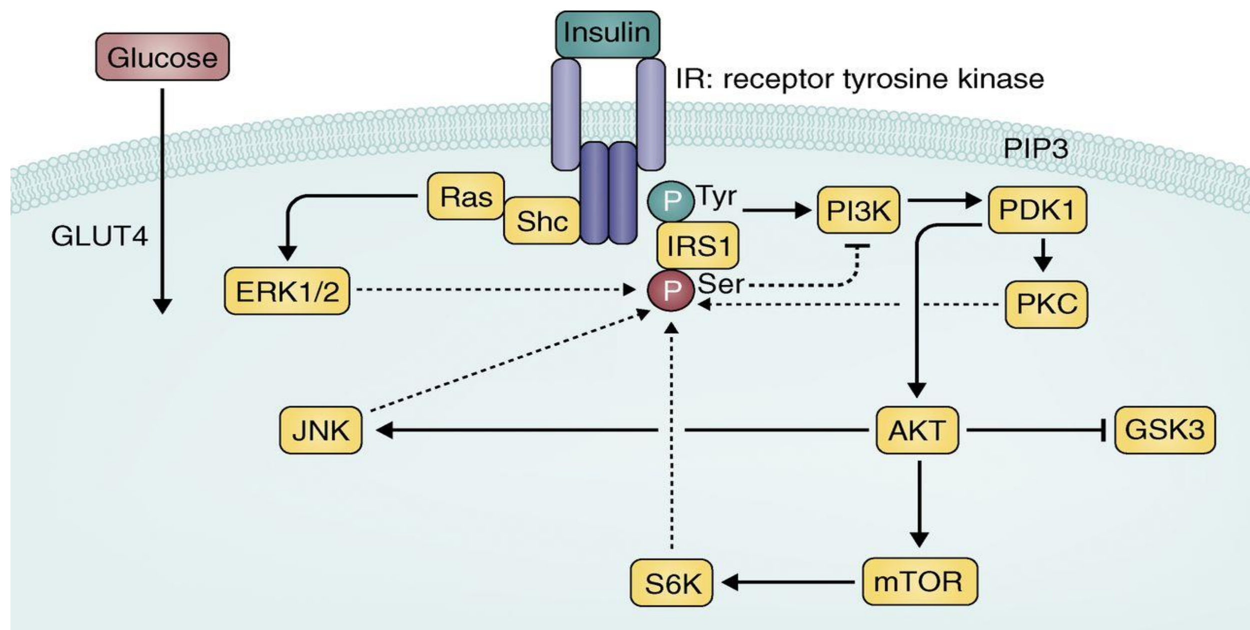

Figure 2: **Illustration of the Insulin signalling pathway** (Molly Stanley et al. 2016.): Insulin binds to the insulin receptor that autophosphorylates and triggers a cascade of phosphorylation events. Phosphorylated on its tyrosine residue, the IRS1 will further activate signalling that eventually leads to the translocation of glucose transporter 4 (GLUT4) to the membrane and uptake of glucose for energy in peripheral tissues. The diagram displays the PI3K pathway. Solid arrows represent activation upon insulin stimulation. Blocked arrows represent inhibition that is thought to lead to less activation of the signalling cascade through negative feedback (dashed blocked arrow).

1.2.3 Phosphatidylinositol-3 kinase (PI3K) pathway

The PI3K pathway depicted in figure 2 is initiated upon insulin binding to the α-subunit of the insulin receptor, which then causes the tyrosine residues in the intracellular β-domain of the insulin receptor to undergo autophosphorylation [40]**.** Thereafter, insulin receptor substrate (IRS) recognizes the activated insulin receptor with its phosphotyrosine binding domain, which subsequently leads to tyrosine phosphorylation and activation of IRS [40]. Once activated, IRS

permits the binding and activation of PI3K that then phosphorylates the membrane lipid phosphatidylinositol (4, 5)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-triphosphate (PIP3). Protein kinase B (PKB, also known as Akt) is recruited to the plasma membrane and activated at PIP3 site in the presence of phosphoinositide-dependant protein kinase-1 (PDK1) [12]. The activation of Akt causes the translocation of the glucose transporter-4 (GLUT-4) to the plasma membrane, thereby promoting glucose uptake. The utilization of blood glucose is accomplished through a process of targeted exocytosis [30]. Simultaneously, GLUT-4 endocytosis is attenuated. In a state of impaired insulin sensitivity, the GLUT-4 slowly recycles between the plasma membrane and vesicular compartments within the cell, where most of the GLUT-4 resides [45]. Therefore, the rate of glucose transport into muscle cells is mostly governed by the concentration of GLUT-4 at the cell surface and the duration for which the protein is maintained at this site [45]. However, the stimulation of glucose uptake by insulin is mediated by PI(3)K–dependent and –independent pathways [46]. In addition, Akt will simultaneously phosphorylate and inactivate glycogen synthase kinase-3 (GSK3) [47]. This results in an increase in glucose storage as glycogen. Glycogen is a branched polymer of glucose kept predominantly in the liver and the skeletal muscle [3], which supply glucose to the bloodstream in the course of fasting periods and to the muscle cells during muscle contraction [48]. The process of glycogen synthesis is dependent on a coordinated action of several enzymes [3].

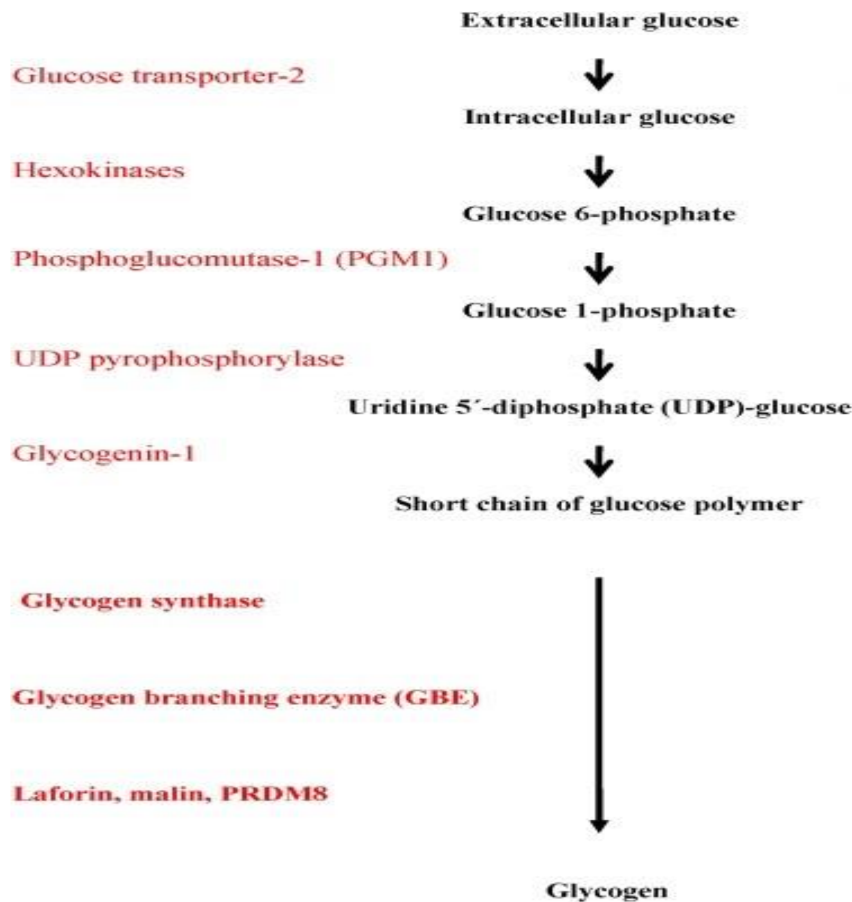

Figure 3: *Glycogen synthesis* (María M. Adeva-Andany, 2016). The diagram depicts the process of glycogen synthesis that is reliant on a coordinated action of several enzymes.

Following the entry of glucose into a cell through glucose transporters, the glucose is phosphorylated to glucose 6-phosphate by hexokinase isoenzymes [49]. Subsequently the isomerization of glucose 6-phosphate into glucose 1-phosphate by phosphoglucomutase-1 [49]. Then, glucose 1-phosphate is converted to uridine 5′-diphosphate (UDP)-glucose in a reaction catalysed by pyrophosphorylase catalyses [49]. Thereafter, the synthesis of glycogen is initiated by glycogenin through autoglycosylation that is transporting glucose from UDP-glucose to itself and making a short linear chain of about 10–20 glucose moieties [49]. And, the elongation of this initial glycogen sequence is catalysed by glycogen synthase that transfers a glycosyl moiety from UDP-glucose to the growing glycogen strand, providing the α-1,4-glycosidic linkages between

glucose residues [50]. Then we have glycogen branching enzyme (GBE) that will introduce branch points in the glycogen particle, by means of creating α-1,6 glycosidic bonds at regular intervals [49]. Therefore, any dysfunctionality in the insulin pathway can lead to metabolic complications such as T2DM. Hence, we sought to assess the gene expression changes that occur in the PI(3)K/Akt insulin signalling pathway of a prediabetic rat model in an attempt to further understand the prediabetic state so as to bring about new innovative means to prevent the onset of T2DM or even reverse it.

1.3 Type 2 Diabetes mellitus

T2DM is a complex disease that is rapidly increasing in prevalence and it is estimated that its prevalence will rise to 439 million by 2030 [2]. Thus, urgent intervention is needed to decrease or even halt the rising prevalence of T2DM. The disease is characterized by hyperglycaemia secondary to insulin resistance (IR) [4]. In addition, T2DM is highly associated with the twenty-first century style of life that consist of a unhealthy high fat-glucose diet, minimal physical activity, shift work and chronic stress that is followed by increased fat storage, systemic IR and substantial elevation in serum insulin, triglyceride and free fatty acid levels [12, 51]. In T2DM conditions, the insulin signalling pathway is generally disturbed and proteins involved in the insulin pathway such as protein kinase beta/Akt, mTOR and GLUT-4 are commonly affected [4, 52]. Thus, this study is interested in looking into these proteins.

1.3.1 Protein Kinase beta/Akt

The serine/threonine-specific protein kinase Akt/PKB is essential in numerous cellular processes, including cell growth, survival, proliferation, and metabolism [51]. According to literature, three

homologous isoforms have been identified and differentiated as Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ [53]. Recent publications have identified discrete roles for each isoform, with Akt1/PKBα linked to cell survival, Akt2/PKBβ with cell-substrate metabolism, and Akt3/PKBγ with brain development [4, 54, 55]. Interestingly, the knockout Akt2/PKBβ mice display elevated glucose levels and reduced glucose transport in muscle, however, these changes are not apparent with deletion of the Akt1/PKBα and Akt3/ PKBγ isoforms [54]. It is evident that Akt signalling plays a fundamental role in insulin-stimulated glucose uptake in skeletal muscle tissue while inhibiting glucose release from hepatocytes. As mentioned above, insulin induces glucose uptake via Akt through stimulated GLUT-4 translocation to the cell membrane where GLUT-4 facilitates glucose uptake [54].

It has been shown from both human and animal work that the presence of IR is associated with defects to both upstream and downstream targets of Akt/PKB in the form of dephosphorylation of protein side chains on IRSs, complete loss of IRSs from the cell surface membrane [56], reduced PI3K activity and impaired phosphorylation of the Akt/PKB substrate AS160 in T2DM skeletal muscle [57]. Furthermore, it has been shown that the depletion of the Akt2/PKBβ isoform in mice results in IR and diabetic-like symptoms, with Akt2/PKBβ knockout rodents also demonstrating hepatic insulin resistance [58]. A study conducted in humans has reported that a mutation in the gene encoding Akt2/PKBβ causes severe IR [54, 59]. Therefore, this establishes Akt2/PKBβ as a key protein in the maintenance of a normal level of sugar in the blood, given that defects in this important mediator would presumably result in reduced AS160-induced GLUT translocation [54]. Therefore, changes in AKT and GLUT-4 gene expression were examined in skeletal muscle harvested from a prediabetic rat model in order to determine the functionality of the insulin signalling pathway at a pre-diabetes level.

1.3.2 mTOR

Akt also activates the mammalian target of rapamycin (mTOR). The activation of mTOR by Akt also promotes fatty acid uptake and synthesis [12]. The mTOR protein is nutrient sensitive and plays a role in mediating the effects of nutrients [60]. Nutrients such as glucose, fatty acids and amino acids are able to independently activate mTOR [61]. In addition, mTOR activity is modulated by repeated contraction (i.e. exercise) and has emerged as an important mediator of exercise-induced adaptation in skeletal muscle [62]. Literature suggests that changes in the activation of the mTOR complexes may be one mechanism to explain the altered insulin signal transduction in response to lipid availability [63]. The mTOR protein is subdivided into two distinct large multi-protein complexes that are mTORC1 and mTORC2 [64]. The present study is particularly interested in the mTORC1 pathway because of its association with insulin resistance when hyperactivated [63]. Upon activation, the mTORC1 increases phosphorylation of ribosomal protein S6 kinase β-1 (p70S6K) and/or eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [65, 66]. In turn, p70S6K induces IR by stimulating the serine-phosphorylation of insulin receptor substratediabetes-1 (IRS-1) that interrupts the recruitment and triggering of PI3K leading to diminished insulin activity [63, 67-69]. Thus, given its role in mediating the effects of nutrients and the capacity for contractile activity to enhance insulin sensitivity and cell growth, mTOR represents an important regulator of skeletal muscle insulin action and metabolism. Consequently, this study investigated the changes in mTORC1 product p70S6K gene expression in a prediabetic state using a prediabetic rat model.

1.3.3 GLUT-4

The glucose transporter type 4 (GLUT-4) is a protein that is dependent on insulin to facilitate glucose uptake in tissues such as the adipose and skeletal muscle. The GLUT-4 is thus a major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis [70, 71]. The importance of GLUT-4 in whole-body metabolism is strongly supported by a variety of genetically engineered mouse models where expression of the transporter is either enhanced or ablated in muscle or adipose tissue or both [72]. The muscle-specific GLUT4 knockout mice characterised with IR is one example that affirms GLUT-4 importance in the insulin pathway [72]. Therefore, this study also seeks to investigate the level of GLUT-4 gene expression during pre-diabetes with the use of a pre-diabetic rat model.

1.4 Pre-diabetes

Pre-diabetes is a chronic metabolic complication that often precedes the onset of T2DM [21, 22]. It can be defined as glycaemic variables that are higher than normal, but lower than diabetes thresholds. Furthermore, pre-diabetes is preceded by insulin resistance (IR) or pancreatic β cell dysfunction way before glucose changes are detectable [22]. Subsequently, pre-diabetes is characterized by moderate hyperglycaemia, hyperlipidaemia, and IR [73]. In the presence of IR, standard insulin levels in the plasma would fail to stimulate a proper insulin response in the peripheral target tissues and as a result the pancreatic β cell responds by producing a greater amount of the hormone insulin in order to overcome the high plasma glucose concentrations amid insulin resistant peripheral target tissues [74, 75]. The upward change in glucose concentrations begins to show when the β cell fails to secrete sufficient insulin to compensate for IR resulting in

overt hyperglycaemia detected in T2DM individuals [76, 77]. Globally it is projected that more than 470 million people will be burdened with pre-diabetes by the year 2030 (5).

Pre-diabetes is determined by observing impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and elevated glycosylated haemoglobin (HbA1c) (15). IFG is defined as fasting plasma glucose (FPG) of 6.1-6.9 mmol/L (110 to 125 mg/dL). IGT is defined as 2 h plasma glucose of 7.8-11.0 mmol/L (140-200 mg/dL) after ingestion of 75 g of oral glucose load or a combination of the two based on a 2 h oral glucose tolerance (OGT) test [78]. The HbA1c represents haemoglobin that is irreversibly glycated at one or both N-terminal valines of the β chains. The HbA1c level provides information about the degree of long-term blood glucose control rather than the exact mean blood glucose [24]. The ADA has proposed a prediabetes diagnosis range of 5.7-6.4% of HbA1c [24].

Pre-diabetes is recognized as the principal risk factor for the development of T2DM with several meta-analytic and pathophysiological studies expressing that the majority onset of T2DM is preceded by pre-diabetes [20-22]. A study conducted in our laboratory showed that the animals fed with HFHC-D developed pre-diabetes as shown by augmented HbA1c and moderate IR [24].

Furthermore, macro/micro-vascular complications seen in overt T2DM have also been detected in the pre-diabetes state [69]. Macrovascular complications comprise of coronary artery disease, peripheral arterial disease, and stroke; and microvascular complications comprise of diabetic nephropathy, neuropathy, and retinopathy [46]. The macrovascular disease is caused by injury to the arteries [79] by the process of atherosclerosis, which cause plaque build-up on the arterial walls leading to the narrowing of arteries throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system [46]. Interestingly, it has been shown that the diabetic and pre-diabetic state is associated with the

release of inflammatory markers like plasma IL-6, TNF-α and cardiac CRP cytokines that ultimately leads to cardiac dysfunction [48, 55, 80]. This is further supported by Jia-liang et al. who showed that transiently elevated blood glucose in impaired glucose tolerance was an indicator of pathological oxidative stress factor in the pre-diabetic state and caused cardiac dysfunction associated with irregular energy metabolism and release of inflammatory factors [81]. In addition, it has been shown that the chances of having a macrovascular disease are upregulated even before glucose levels reach the diagnostic threshold for diabetes [82]. Furthermore, one-fourth of newly diagnosed diabetics are already shown to have overt cardiovascular disease [69, 82].

However, the risk of developing microvascular complications of diabetes depends on both the period and the severity of hyperglycaemia. But these complications as well may occur prior to overt T2DM, for example, some literature claim that 8% of a population diagnosed with pre-diabetes already have microvascular complications such as retinopathy [83] and that retinopathy foretells subsequent risk of diabetes [84]. The aldose reductase is regarded as the possible pathological mechanisms leading to the progression of microvascular complications [85]. In the intracellular polyol pathway where aldose reductase acts as the initial enzyme, glucose is converted into glucose alcohol (sorbitol). Thus, high glucose levels increase the flux of sugar molecules through the polyol pathway, which causes sorbitol accumulation in cells [85]. Osmotic stress from sorbitol accumulation has been postulated as an underlying mechanism in the development of diabetic microvascular complications. In addition, high glucose concentrations can promote the nonenzymatic formation of advanced glycosylated end products (AGEs) [46]. Oxidative stress may also play an important role in cellular injury from hyperglycaemia. High glucose levels can stimulate free radical production and reactive oxygen species formation [46, 86]. Therefore, since diabetic complications begin prior to the onset of diabetes mellitus, it is essential that the pre-

diabetic condition is looked at more extensively in order to prevent the common progression of pre-diabetes to T2DM.

1.5 Justification

Literature documents that the onset of overt T2DM is often preceded by pre-diabetes [21]. Therefore, it is of utmost importance to stress the necessity of having extra attention directed towards the investigation of the pre-diabetes condition, for "prevention is better than cure". Therefore, this study was conducted based on that no work has been conducted in analysing the possible alterations in the expression of genes coding for proteins involved in the insulin pathway during the pre-diabetic state. The target genes consist of Akt, p70S6K & GLUT-4 and they were chosen based on literature findings mentioned above and their involvement in the insulin signalling pathway. Findings, therefore, will possibly reveal whether the insulin pathway experiences any alterations in the prediabetic model utilized and will broaden understanding of the mechanism leading to insulin resistance and its link with genetics in HFHC-D induced pre-diabetes. Furthermore, findings will assist in the quest to establish an effective strategy for pharmacological therapy targeting diabetes/pre-diabetes. This could be through identifying distinctive features of pre-diabetes and subsequently discover ways to selectively target them.

1.6 Aim & Objectives

However, the mechanics leading to the induction of pre-diabetes characterized by IR have not yet been studied in this model. Thus, the aim of the study was to investigate the changes in insulin signalling pathway of the pre-diabetes model.

This was achieved by:

- Examining the OGT test following the induction of pre-diabetes with a high fat high carbohydrate diet.
- Measuring the HOMA2-IR which determines insulin sensitivity.
- Determining the level of expression for genes that code for the proteins of interest associated with the insulin pathway.

1.7 List of references

1. Radha V, Vimaleswaran KS, Deepa R, Mohan V. The genetics of diabetes mellitus. The Indian journal of medical research. 2003;117:225-38.

2. Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. Oman medical journal. 2012;27(4):269.

3. Bouché C, Serdy S, Kahn CR, Goldfine AB. The Cellular Fate of Glucose and Its Relevance in Type 2 Diabetes. Endocrine Reviews. 2004;25(5):807-30.

4. Kahn S. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. Diabetologia. 2003;46(1):3-19.

5. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014;37(Supplement 1):S81-S90.

6. Chaudhury A, Duvoor C, Dendi R, Sena V, Kraleti S, Chada A, et al. Clinical review of antidiabetic drugs: Implications for type 2 diabetes mellitus management. Frontiers in endocrinology. 2017;8:6.

7. Lizcano JM, Alessi DR. The insulin signalling pathway. Current biology. 2002;12(7):R236-R8.

8. 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;27(5):1047-53.

9. Roglic G. WHO Global report on diabetes: A summary. International Journal of Noncommunicable Diseases. 2016;1(1):3.

10. Organization WH. Diabetes country profiles https://www.who.int/ 2016.

11. 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;27(5):1047-53.

12. Lv J, Yu C, Guo Y, Bian Z, Yang L, Chen Y, et al. Adherence to a healthy lifestyle and the risk of type 2 diabetes in Chinese adults. International journal of epidemiology. 2017;46(5):1410-20.

13. Odermatt A. The Western-style diet: a major risk factor for impaired kidney function and chronic kidney disease. American Journal of Physiology-Renal Physiology. 2011;301(5):F919-F31.

14. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. The Journal of clinical investigation. 2008;118(3):829-38.

15. Reynoso R, Salgado LM, Calderón V. High levels of palmitic acid lead to insulin resistance due to changes in the level of phosphorylation of the insulin receptor and insulin receptor substrate-1. Vascular Biochemistry: Springer; 2003. p. 155-62.

16. Steyn NP, Mann J, Bennett P, Temple N, Zimmet P, Tuomilehto J, et al. Diet, nutrition and the prevention of type 2 diabetes. Public health nutrition. 2004;7(1a):147-65.

17. Kovacs P, Hanson RL, Lee Y-H, Yang X, Kobes S, Permana PA, et al. The Role of Insulin Receptor Substrate-1 Gene (IRS1) in Type 2 Diabetes in Pima Indians. Diabetes. 2003;52(12):3005-9.

18. Song C, Liu D, Yang S, Cheng L, Xing E, Chen Z. Sericin enhances the insulin-PI3K/AKT signaling pathway in the liver of a type 2 diabetes rat model. Experimental and therapeutic medicine. 2018;16(4):3345-52.

19. Yao XH, Nguyen KH, Nyomba BG. Reversal of glucose intolerance in rat offspring exposed to ethanol before birth through reduction of nuclear skeletal muscle HDAC expression by the bile acid TUDCA. Physiological reports. 2014;2(12).

20. Xu J-T, Zhao X, Yaster M, Tao Y-X. Expression and distribution of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in rat dorsal root ganglion and spinal cord dorsal horn. Brain research. 2010;1336:46-57.

21. Forouhi N, Luan J, Hennings S, Wareham N. Incidence of type 2 diabetes in England and its association with baseline impaired fasting glucose: the Ely study 1990–2000. Diabetic Medicine. 2007;24(2):200-7.

22. Tabák AG, Herder C, Rathmann W, Brunner EJ, Kivimäki M. Prediabetes: a high-risk state for diabetes development. The Lancet. 2012;379(9833):2279-90.

23. Yip WCY, Sequeira IR. Prevalence of Pre-Diabetes across Ethnicities: A review of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) for classification of dysglycaemia. 2017;9(11).

24. Mluleki Luvunyo MM, Andile Khathi. Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. Ponte Academic Journal 2017;74(5).

25. Mabuza LP, Gamede MW, Maikoo S, Booysen IN, Ngubane PS, Khathi A. Effects of a Ruthenium schiff base complex on glucose homeostasis in diet-induced pre-diabetic rats. Molecules. 2018;23(7):1721.

26. Gamede M, Mabuza L, Ngubane P, Khathi A. The effects of plant-derived oleanolic acid on selected parameters of glucose homeostasis in a diet-induced pre-diabetic rat model. Molecules. 2018;23(4):794.

27. Engelking L, Rebar AH. Metabolic and endocrine physiology: CRC Press; 2012.

28. Qaid MM, Abdelrahman MM. Role of insulin and other related hormones in energy metabolism—A review. Cogent Food & Agriculture. 2016;2(1):1267691.

29. Squires E. Manipulation of growth and carcass composition. Applied animal endocrinology. 2010(Ed. 2):89-158.

30. Lee J, Pilch P. The insulin receptor: structure, function, and signaling. American Journal of Physiology-Cell Physiology. 1994;266(2):C319-C34.

31. Nussey S, Whitehead S. Endocrinology: an integrated approach. BIOS Scientific: BIOS Scientific Publishers Limited; 2001. 358 p.

32. Wilcox, Gisela. “Insulin and insulin resistance” Clinical biochemist. Reviews vol. 26,2 (2005): 19-39.

33. Peti W, Page R. Molecular basis of MAP kinase regulation. Protein science. 2013;22(12):1698-710.

34. Arthur JSC, Ley SC. Mitogen-activated protein kinases in innate immunity. Nature Reviews Immunology. 2013;13(9):679.

35. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiological reviews. 2012;92(2):689-737.

36. Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases—regulating the immune response. Nature Reviews Immunology. 2007;7(3):202.

37. Owens D, Keyse S. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene. 2007;26(22):3203.

38. Soares-Silva M, Diniz FF, Gomes GN, Bahia D. The mitogen-activated protein kinase (MAPK) pathway: role in immune evasion by trypanosomatids. Frontiers in microbiology. 2016;7:183.

39. Yang R, Trevillyan JM. c-Jun N-terminal kinase pathways in diabetes. The International Journal of Biochemistry & Cell Biology. 2008;40(12):2702-6.

40. Saltiel AR, Pessin JE. Insulin signaling pathways in time and space. Trends in Cell Biology. 2002;12(2):65-71.

41. Saltiel AR, Pessin JE. Insulin Signaling in Microdomains of the Plasma Membrane. Traffic. 2003;4(11):711-6.

42. Prada PO, Pauli JR, Ropelle ER, Zecchin HG, Carvalheira JBC, Velloso LA, et al. Selective modulation of the CAP/Cbl pathway in the adipose tissue of high fat diet treated rats. FEBS Letters. 2006;580(20):4889-94.

43. Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, et al. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature. 2000;407(6801):202-7.

44. Chiang S-H, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, et al. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature. 2001;410:944.

45. Chang L, Chiang S-H, Saltiel AR. Insulin signaling and the regulation of glucose transport. Molecular medicine. 2004;10(7-12):65.

46. Fowler MJ. Microvascular and macrovascular complications of diabetes. Clinical diabetes. 2008;26(2):77-82.

47. Mukundwa A, Mukaratirwa S, Masola B. Effects of oleanolic acid on the insulin signaling pathway in skeletal muscle of streptozotocin-induced diabetic male Sprague-Dawley rats. Journal of diabetes. 2016;8(1):98-108.

48. Makki K, Froguel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. International Scholarly Research Notices (ISRN) inflammation. 2013;2013.

49. Adeva-Andany MM, González-Lucán M, Donapetry-García C, Fernández-Fernández C, Ameneiros-Rodríguez E. Glycogen metabolism in humans. BBA Clinical. 2016;5:85-100.

50. Roach PJ. Control of glycogen synthase by hierarchal protein phosphorylation. The FASEB Journal. 1990;4(12):2961-8.

51. Morris CJ, Yang JN, Garcia JI, Myers S, Bozzi I, Wang W, et al. Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans. Proceedings of the National Academy of Sciences. 2015:201418955.

52. Saini V. Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. World journal of diabetes. 2010;1(3):68-75.

53. Schinner S, Scherbaum W, Bornstein S, Barthel A. Molecular mechanisms of insulin resistance. Diabetic Medicine. 2005;22(6):674-82.

54. Mackenzie RWA, Elliott BT. Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2014;7:55-64.

55. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 2007;132(6):2169-80.

56. Zick Y. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. Trends in Cell Biology. 2001;11(11):437-41.

57. Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, Wallberg-Henriksson H. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. Diabetes. 2005;54(6):1692-7.

58. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, et al. Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ). Science. 2001;292(5522):1728-31.

59. George S. A Family with Severe Insulin Resistance and Diabetes Mellitus due to a Missense Mutation in AKT2. 2004;304(5675):1325-8.

60. Tremblay Fdr, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146(3):1328-37.

61. C Melnik B. The pathogenic role of persistent milk signaling in mTORC1-and milk-microRNA-driven type 2 diabetes mellitus. Current diabetes reviews. 2015;11(1):46-62.

62. Coffey VG, Hawley JA. The molecular bases of training adaptation. Sports medicine. 2007;37(9):737-63.

63. Rivas DA, Yaspelkis BB, Hawley JA, Lessard SJ. Lipid-induced mTOR activation in rat skeletal muscle reversed by exercise and 5′-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside. Journal of Endocrinology. 2009;202(3):441-51.

64. Vergès B. mTOR and cardiovascular diseases: diabetes mellitus. Transplantation. 2018;102(2S):S47-S9.

65. Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR, et al. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. The Journal of physiology. 2003;553(1):213-20.

66. Kubica N, Bolster DR, Farrell PA, Kimball SR, Jefferson LS. Resistance exercise increases muscle protein synthesis and translation of eukaryotic initiation factor 2Bϵ mRNA in a mammalian target of rapamycin-dependent manner. Journal of Biological Chemistry. 2005;280(9):7570-80.

67. Vergès B, Cariou B. mTOR inhibitors and diabetes. Diabetes research and clinical practice. 2015;110(2):101-8.

68. Vergès B. mTOR and Cardiovascular Diseases: Diabetes Mellitus. Transplantation. 2017. ;102: S47–S49.

69. Blagosklonny M. TOR-centric view on insulin resistance and diabetic complications: perspective for endocrinologists and gerontologists. Cell death & disease. 2013;4(12):e964.

70. Joost H-G, Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members. Molecular membrane biology. 2001;18(4):247-56.

71. Wood IS, Trayhurn P. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. British journal of nutrition. 2003;89(1):3-9.

72. Huang S, Czech MP. The GLUT4 glucose transporter. Cell metabolism. 2007;5(4):237-52.

73. Karpovets T, Konopelnyuk V, Galenova T, Savchuk A, Ostapchenko L. High-calorie diet as a factor of prediabetes development in rats. Bulletin of experimental biology and medicine. 2014;156(5):639.

74. Wang G. Raison d'être of insulin resistance: the adjustable threshold hypothesis. Journal of the Royal Society, Interface. 2014;11(101):20140892-.

75. Gill H, Mugo M, Whaley-Connell A, Stump C, Sowers JR. The key role of insulin resistance in the cardiometabolic syndrome. The American journal of the medical sciences. 2005;330(6):290-4.

76. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes. 2004;53(suppl 3):S16-S21.

77. Tabák AG, Jokela M, Akbaraly TN, Brunner EJ, Kivimäki M, Witte DR. Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. The Lancet. 2009;373(9682):2215-21.

78. Nathan DM, Davidson MB, DeFronzo RA, Heine RJ, Henry RR, Pratley R, et al. Impaired Fasting Glucose and Impaired Glucose Tolerance. Implications for care. 2007;30(3):753-9.

79. Forbes JM, Coughlan MT, Cooper ME. Oxidative stress as a major culprit in kidney disease in diabetes. Diabetes. 2008;57(6):1446-54.

80. Emanuela F, Grazia M, Marco DR, Maria Paola L, Giorgio F, Marco B. Inflammation as a link between obesity and metabolic syndrome. Journal of nutrition and metabolism. 2012;2012.

81. Liang J-L, Feng Z-K, Liu X-Y, Lin Q-X, Fu Y-H, Shan Z-X, et al. Effect of impaired glucose tolerance on cardiac dysfunction in a rat model of prediabetes2011. 734-9 p.

82. Wilson PW, Kannel WB. Obesity, diabetes, and risk of cardiovascular disease in the elderly. The American journal of geriatric cardiology. 2002;11(2):119-24.

83. Group DPPR. The prevalence of retinopathy in impaired glucose tolerance and recent-onset diabetes in the Diabetes Prevention Program. Diabetic Medicine. 2007;24(2):137-44.

84. Wong T, Mohamed Q, Klein R, Couper D. Do retinopathy signs in non-diabetic individuals predict the subsequent risk of diabetes? British journal of ophthalmology. 2006;90(3):301-3.

85. Gabbay KH. Hyperglycemia, polyol metabolism, and complications of diabetes mellitus. Annual review of medicine. 1975;26(1):521-36.

86. Fong DS, Aiello LP, Ferris FL, Klein R. Diabetic retinopathy. Diabetes care. 2004;27(10):2540-54.

Chapter 2 (Manuscript)

Prologue

Type 2 diabetes is regarded as a global crisis by the world health organisation (WHO). However, it is often preceded by a prediabetic or intermediate hyperglycaemic condition. Recently published work conducted in our laboratory successfully developed a pre-diabetes rat model characterized with moderate insulin resistance (IR) through a diet high in fat and carbohydrates. But no work has been done with this model to disclose the mechanics that lead to pre-diabetes. Therefore, we targeted the insulin pathway as the possible pathway that is disturbed in the process of developing pre-diabetes and subsequently T2DM. Consequently, we investigated the changes that occur in the insulin signalling pathway of a rat prediabetic model by measuring the genetic and protein expression of hormones involved in the pathway. The manuscript has been formatted and submitted according to the PLOS ONE submission guidelines (appendix 3).

Title

Investigating changes to the insulin signalling pathway in a diet-induced pre-diabetic rat model: Effects on selected markers

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Abstract

Pre-diabetes is a chronic metabolic complication that often precede the onset of type two diabetes mellitus and can be defined as glycaemic variables that are higher than normal, but lower than diabetes thresholds, and is preceded by insulin resistance. The extent of changes occurring in the expression of genes coding for proteins involved in insulin signalling pathway in a pre-diabetes rodent model fed with a high-fat high-carbohydrate diet (HFHC-D) has not been explored. Therefore, following the induction of pre-diabetes with HFHC-D, biochemical analysis was conducted. The enzyme-linked immunosorbent assay (ELISA) rat insulin kit was used to assess insulin levels and real time-quantitative Polymerase chain reaction (RT-qPCR) technique was used to measure the gene expression of specific proteins in the skeletal muscle that have an influence in the insulin pathway. The pre-diabetes group fed with HFHC-D experienced weight gain due to its high calorie intake and was characterized with elevated HOMA-IR index, triglycerides and AUC levels of the OGT curve. The results show an elevated expression of the protein kinase B/Akt and protein S6 kinase (p70S6) genes. Meanwhile, glucose transporter 4 (GLUT-4) gene was diminished. This suggest that the insulin pathway is already partially distorted in the pre-diabetes state due to the role of p70S6 at inhibiting the insulin pathway at the IRS-1. This is evidenced by the minimal expression of glucose transporter 4. Therefore, the popularization of diet intervention and pre-diabetes specific treatment is essential to reduce rising prevalence of type two diabetes mellitus.

Keywords: Type two diabetes mellitus, pre-diabetes, insulin resistance, gene expression,

1.0 Introduction

Type 2 Diabetes Mellitus (T2DM) is an intricate disease that has become pandemic and unless an intervention is found, incident cases are expected to continue to rise [1, 2]. The disease is characterized by hyperglycaemia secondary to insulin resistance (IR) [3, 4]. In addition, T2DM is associated with lifestyle risks factors such as living a sedentary lifestyle and the consumption of an unhealthy diet [5]. Furthermore, these risk factors have been implicated in changes in expression of genes associated with the insulin signalling pathway [6]. However, the onset of T2DM is often preceded by a long lasting condition known as pre-diabetes [7, 8]. Pre-diabetes is defined as glycaemic variables that are higher than normal, but lower than diabetes thresholds, and is preceded by IR [8]. A study conducted in our laboratory showed that the animals fed with a high-fat high-carbohydrates diet (HFHC-D) developed pre-diabetes as shown by augmented glycosylated haemoglobin and moderate IR [9-11].

The insulin signalling pathway is one of the processes involved in the regulation of glucose homeostasis by facilitating the entry of glucose into tissues such as the skeletal muscle for either to be used as fuel or stored as glycogen [12-14]. This is achieved by the activation of a downstream target of the insulin pathway known as protein kinase B (PKB, also known as Akt) that in turn phosphorylates and inactivates glycogen synthase kinase-3 (GSK3), an enzyme involved in glycogen synthase phosphorylation and inactivation resulting in an increase in glucose storage as glycogen [5, 15]. Akt is also responsible for the translocation of the glucose transporter-4 (GLUT-4) to the plasma membrane, thereby promoting glucose uptake [5]. Finally, Akt is responsible for the activation of the mTORC1 protein that results in protein synthesis and fatty acid synthesis [16].

However, in T2DM the insulin pathway experience changes such as the continual activation of mTORC1 that stimulates S6K1, which, in turn, inhibits insulin receptor signalling at the cellular

membrane [17, 18]. In addition, the phosphoinositide 3-kinase (PI3K) and Akt are not activated in T2DM, resulting to reduced cellular glucose uptake that is characterized by a diminished activation of glycogen synthase, and decrease in the proportion of GP in the active phosphorylated form [12, 19]. But, in the prediabetic state, it is not clear whether these changes already exist and/or whether there are alterations in gene expression coding for proteins involved in the insulin pathway. Therefore, we sought to investigate the changes that occur in the insulin signalling pathway and in gene expression of proteins involved in the insulin pathway utilizing a pre-diabetes rat model.

2.0 Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents were of analytical grade and were purchased from standard commercial suppliers.

2.2. Animals

Male Sprague-Dawley rats (150-250g) bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content (<5000 p.m.), relative humidity (55±5%) and illumination (12 hr light/dark cycle, lights on at 07h00). The animals were allowed access to food and fluids ad libitum. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Ethics number: AREC/026/017M; see appendix 1). The animals were allowed to acclimatize to their new

environment for about 1 week while consuming standard rat chow and tap water before exposure to the experimental diets. Procedures involving animals care were conducted in conformity with the institutional guidelines for animal care of the University of KwaZulu-Natal.

2.3. Experimental diet

A diet high in fats and carbohydrates was chosen because it resembles the current mode of life with high fat glucose diet. The composition of a high fat high carbohydrate diet was customized as follows: carbohydrates (55 % Kcal/g), fats (30 % Kcal/g), and proteins (15 % Kcal/g). Further description of the diet can be seen in table 1 & 2. The drinking water of the experimental animals was supplemented with 15% fructose and 5% powder milk. The details for the normal diet can be seen in table 3.

Table 1: Composition of the high fats high carbohydrates (HFHC) diet

Ingredient	Incl(%)	Mix(kg)
Maize	38.98	390.000
Palm Oil	20.99	210.000
Soya Full Fat	14.99	150.000
Wheat Gluten	6.50	65.000
Flour	6.00	60.000
Monodex	5.00	50.000
Sugar – White	5.00	50.000
Limestone	1.00	10.000

Dicalcium Phosphate	0.50	5.000
Vitamin Premix	0.35	3.500
Salt – Fine	0.30	3.000
Amino Acid - DL Methionine	0.30	3.000
Mineral Premix	0.10	1.000
	100.01	**1000.50**

Table 2: Nutritional value of the high fats high carbohydrates (HFHC) diet

Nutrient	**Units**	**Actual**
Dry Matter	g/kg	919.93
Metabolizable Energy	MJ/kg	15.86
Crude Protein	g/kg	151.27
AShreonine	g/kg	4.51
ASIsoluecine	g/kg	5.24
ASLysine	g/kg	6.54
ASMethionine	g/kg	4.86
ASryptophan	g/kg	1.30
ASstidine	g/kg	3.30
ASTSAA	g/kg	6.79

ASValine	g/kg	5.80
Fat	g/kg	250.46
Carbohydrate	g/kg	427.29
Fibre	g/kg	22.08
Ash	g/kg	26.31
Avl Phosphorus	g/kg	1.66
Calcium	g/kg	5.47
Total Phosphorus	g/kg	3.60

Table 3: Composition of fats, proteins and carbohydrates of the normal diet (ND).

Fats	15 %
Proteins	25 %
Carbohydrates	65 %

2.4. Experimental protocol

Male Sprague-Dawley rats (150-250g) were randomly assigned to the following diet groups (n= 4 per group): a standard rat chow diet with normal drinking water (ND) and high-fat high-carbohydrate diet with drinking water supplemented with 15% fructose and 5% nestle powder milk (HFHC-D), The experimental period was >20 weeks including diet adaptation periods. Body weights, food intake/calorie intake, total triglyceride concentrations, and as well as oral glucose

tolerance (OGT) responses were measured in the animals during the experimental period until the termination. The animals were sacrificed after the experimental period to remove the skeletal muscle for biochemical analysis and collect blood samples for plasma insulin concentration measurements.

2.5. Oral glucose tolerance (OGT) response

Performed to determine the glucose tolerance response of animals subjected to a chronic ingestion of a high-fat high-carbohydrate diet (>20 weeks), an oral glucose tolerance (OGT) test was conducted following carbohydrate loading. The OGT response of all animal groups was monitored in the animals using our established laboratory protocol (21). Briefly, after an 12 h fasting period, glucose levels were measured (time 0) followed by loading with a monosaccharide syrup (glucose; 0.86 g/kg) by oral gavage using an 18-gauge gavage needle that is 38 mm long curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). To measure glucose concentration, blood was collected using the tail-prick method (22). Glucose concentrations were measured by a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose concentrations were measured at 15, 30, 60, and 120 minutes following carbohydrate loading.

2.6. Blood collection and tissue harvesting

For blood collection, all animals were anaesthetised with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for ~2 minutes. While rats were unconscious, they were decapitated, and blood was collected and injected into individual heparinized and EDTA

containers. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. Plasma was collected and stored at -80 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until ready for biochemical analysis. Thereafter, the skeletal muscle was removed, rinsed with cold normal saline solution and snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C for biochemical analysis.

2.7. Glycogen Assay

Glycogen analysis was performed in muscle tissues using a well-established laboratory protocol [20, 21]. The harvested muscle tissues were weighed and heated with potassium hydroxide (KOH) (30%, 2 mL) at 100 °C for 30 min. Then immediately disodium sulphite (10%, 0.194 mL) was added in to the mixture to stop the reaction. The mixture was then allowed to cool, and the glycogen precipitate was formed. The cooled mixture with precipitate was aspirated (200 µL) and mixed with ethanol (95%, 200 µL). The precipitated glycogen was pelleted, washed and resolubilized in water (1 mL). Thereafter, anthrone (0.5 g dissolve in 250 mL of sulphuric acid, 4 mL) was added and boiled for 10 min. After cooling the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany) at 620 nm. The glycogen concentrations were calculated from the glycogen standard curve.

2.8 HOMA2-IR index

The homeostatic model assessment (HOMA) is an authenticated technique utilised to measure IR from fasting glucose and insulin [22]. The HOMA consist of two models identified as either HOMA1-IR or HOMA2-IR. The HOMA2-IR index was the model of choice because it was

updated with some physiological adjustments to a computer version yielding a more accurate index relative to the initial HOMA1-IR that was published by Mattews and cols. in 1985 [23]. A range of 0.5-1.4 is an indication of a normal HOMA-IR value. An index value that is less than 1.0 means that the insulin-sensitivity is at optimum. Whereas, index values above 1.9 indicate early insulin resistance and for index values above 2.9 indicate significant insulin resistance. Therefore, the study utilised the HOMA2-IR index in order to assess the insulin sensitivity in Sprague Dawley rats.

2.9. Biochemical analysis

2.9.1 Plasma insulin

Plasma insulin concentrations were measured using an ultra-sensitive rat insulin ELISA kit (Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden) according to the manufacturer's instructions.

2.9.2 Real Time quantitative PCR (RT-qPCR)

RNA was extracted from the harvested skeletal muscle tissue using a ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, USA). Total RNA (1 μg) was reverse-transcribed into cDNA using a GoTaq® 2-Step RT-qPCR System as a cDNA synthesis kit (Promega, USA) following the supplier's instructions.

The ROCHE light cycler SYBR Green I master mix was used to carry out PCR amplifications on ROCHE light cycler 96 machine. The Primer sequence used can be found in table 4 below. PCR was done using the following cycling conditions: Pre-incubation for 10 min at 95 °C, followed by

45 cycles of 95 °C for 15 s, 60 °C for 30s and 72 °C for 30s. The qRT-PCR results were analysed using the $2^{-\Delta\Delta Cq}$ comparative method to compare Cq values of the treated groups to the control group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used to normalise the Cq values of the treated groups and control groups. To calculate the fold change, a value of 1 was assigned to the controls [24].

Table 4: List of primers used in the study

Sequence name	Sequence
AKT gene	Forward: 5′-ATGGACTTCCGGTCAGGTTCA-3′ Reverse: 5′-GCCCTTGCCCAGTAGCTTCA-3′
GLUT-4	Forward: 5′-ATCAACGCCCCACAGAAAGT-3′ Reverse: 5′-CCTGCCTACCCAGCCAAGT-3′
P70S6	Forward: 5′-GGAGCCTGGGAGCCCTGATGTA-3′ Reverse: 5′-GAAGCCCTCTTTGATGCTGTCC-3′
GAPDH	Forward: 5’-AGTGCCAGCCTCGTCTCATA-3’ Reserve: 5’-GATGGTGATGGGTTTCCCGT-3’

2.10. Analysis of data

Data was expressed as means ± S.E.M. Statistical comparisons were performed with GraphPadInStat Software (version 5.00, GraphPad Software, Inc., San Diego, California, USA)

using the unpaired t-test (Mann-Whitney test) to determine statistical differences between the means of the two groups. A value of $p < 0.05$ was considered statistically significant.

3.0 Results

3.1 Calorie intake and Body weights

The table displays the calorie intake and body weights measured in the final week for both the ND and HFHC-D groups. The calorie intake along with the body weight for the HFHC-D group was significantly higher when compared to that of the ND group ($p < 0.05$).

Table 5: Calorie intake and body weight. Values presented as mean and SEM, * = $p < 0.05$ denotes comparison with ND. For body weights p < 0,0043 .

Groups	**Calorie intake (kcal/g)**	**Body Weights (g)**
ND	106.18±1.90	426.00 ±15.57
HFHC-D	120.72±1.01*	542.30 ±12.47*

3.2 Oral Glucose Tolerance Test

The OGTT was conducted at the culmination of the diet induced pre-diabetes period in ND and HFHC-D groups. The results display that there was no significance difference between the ND and HFHC-D groups during the OGT response. However, there was a significance difference in the Area under the curve (AUC) between the ND and HFHC-D groups.

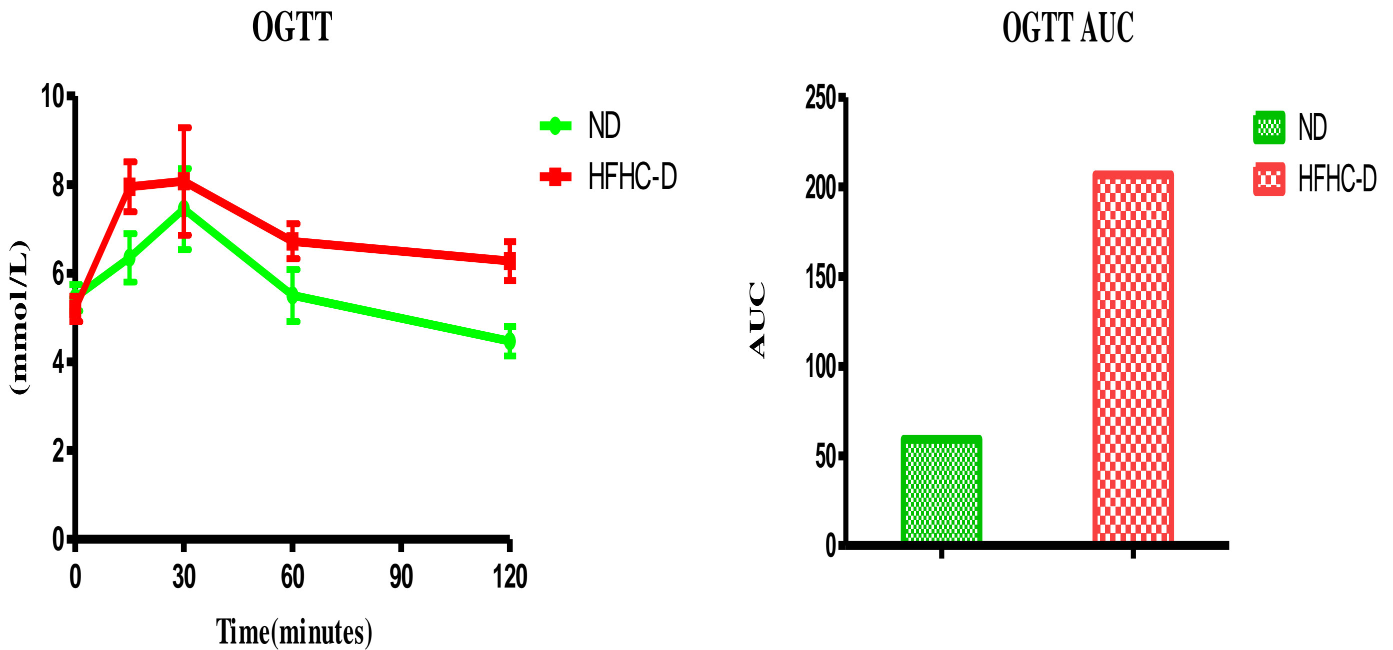

Figure 1 The OGT response after induction of pre-diabetes and its AUC values. Findings show no significance difference between the ND and HFHCID groups during the 120-minute interval. However, the AUC for the HFHC-D group is about four times greater than the ND group. Values presented as mean and SEM (n=4, per group).

3.3 Skeletal muscle glycogen concentrations

The glycogen concentration levels were measured at the terminal of diet induced pre-diabetes period. The results displayed that HFHC-D group had significantly higher skeletal muscle glycogen in comparison to ND ($p < 0.05$).

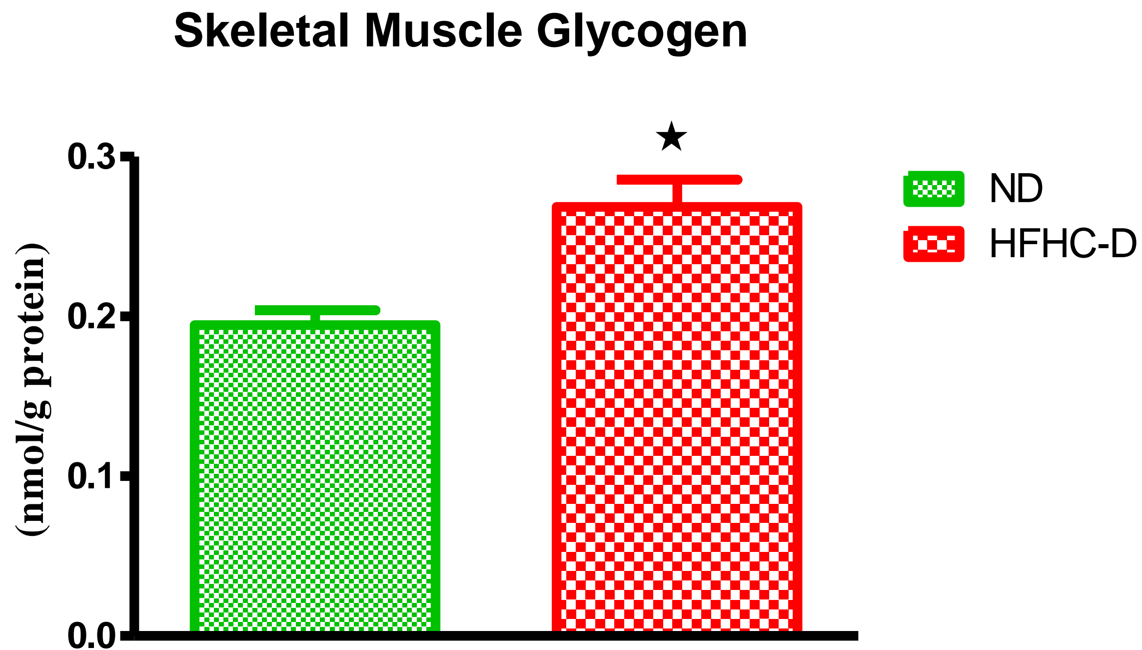

Fig 2. Skeletal muscle glycogen concentrations. Displays terminal muscle glycogen concentration of ND and HFHC-D groups. Values presented as mean and SEM (n=4, per group). ⋆ denotes HFHC-D increased by p < 0,0066 when compared with ND.

3.4 HOMA2-IR Index

The HOMA2-IR index as calculated in the ND and HFHC-D groups. The HOMA2-IR value of the ND group was within insulin-sensitivity range (<1.0). By comparison with the ND group, the HFHC-D group showed a higher HOMA2-IR value, which was above the early IR range (>1.9) but lower than the overt IR range (>2.9). (* = IR range > 1.9).

Table 6: Displays the HOMA2-IR index calculated using the HOMA2 calculator in the ND & HFHC-D groups (n=4, per group).

GROUPS	GLUCOSE (mmol/l)	INSULIN (pmol/L)	HOMA2-IR
ND	5.45	42.27	0.81
HFHC-D	5.20	161.10	2.40 *

3.5 Blood Triglycerides levels

Triglycerides levels was measured in the blood using the Accutrend Plus instrument. In comparison with the ND, there is a significant increase in triglycerides levels in the HFHC-D fed group ($p < 0.05$).

Table 7: The effects of HFHC-D on Triglycerides levels of Sprague Dawley rats during the pre-diabetes induction period. Values presented as mean and SEM (n=4, per group). *=p ≤0,0040.

GROUPS	TG (mmol/l)
ND	1.41 ±0.24
HFHC-D	5.40 ±0.86 *

3.6 Gene expression

The expression of the three genes of interest (GLUT-4, Akt & P70S6) was measured using a real time quantitative polymerase chain reaction (RT-qPCR). The results show that GLUT-4 gene remained relatively the same as there was no increase. However, Akt expressed a 4-fold increase in gene expression and P70S6 expressed a 2-fold increase in its gene expression.

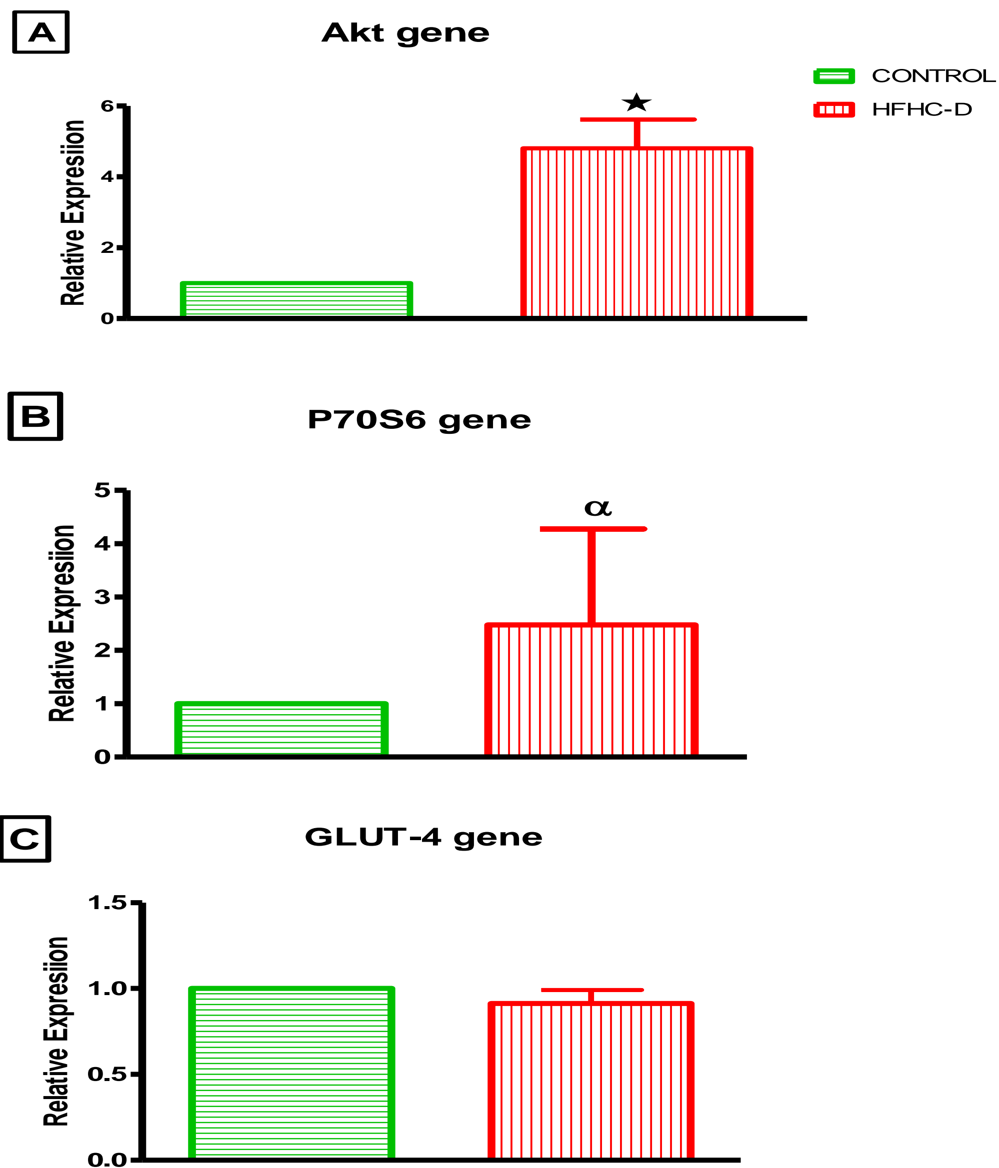

Fig 2. The gene expression of Akt (A), P70S6K (B) and GLUT-4 (C). Bar graph representing the expression (2-ΔCt) of three genes of interest expressed in the skeletal muscle of diet induced prediabetic Sprague Dawley rats. Values presented as mean and SEM, *=p <0.05 and α=p>0.05 denotes vs Normal diet (ND). Akt p≤ 0,0421; P70S6K p≤ 0,4982; GLUT-4 p≤ 0,384. GAPDH was utilized as the housekeeping gene

4.0 Discussion

This study investigated the possible alterations in the insulin signaling pathway in a HCHF-D induced pre-diabetes Sprague Dawley rat model. The model has shown to display the signs and symptoms of pre-diabetes, which include moderate IR and glucose intolerance over time [9, 10]. Also, in literature pre-diabetes is known to be characterized by moderate hyperglycaemia, hyperlipidaemia, and IR [25].

Pre-diabetes is also described as a combination of excess body fat and insulin resistance, thus its associated with unhealthy eating and weight gain/obesity [26, 27]. Unsurprisingly, the animals fed with the high fat high carbohydrates diet experienced a higher calorie input and thus more weight gain relative to normal/standard diet fed group. This weight gain is expected during pre-diabetes because in the prediabetic state, insulin resistant is still at a moderate level. Therefore, insulin is still effective due to the compensatory mechanism of the pancreatic beta cells that release more insulin into the blood and insulin being an anabolic hormone endorses the storage of glucose as glycogen and fat build up in the body [13, 28]. The outcome of this is inevitably the gain of weight.

According to the American Diabetes Association (ADA), pre-diabetes mellitus can be diagnosed with a fasting plasma glucose levels of 5.6 to 6.9 mmol/L and with plasma glucose levels of 7.8

to 11.0 mmol/L 2-hour postprandial, or a glycated haemoglobin (HbA1c) of 5.7 to 6.4% [29] OGT test has been extensively used as a diagnostic tool to screen for diabetes [30]. However, the utilization of OGT test as a diagnostic tool for pre-diabetes is debatable due to its poor reproducibility [31, 32]. Subsequently, HbA1c has been the most widely used and accepted test for monitoring the glycaemic control in individuals with diabetes. Recently published studies conducted in our laboratory using the same HFHC-D induced pre-diabetes rat model has already shown that increased levels of HbA1c are present in the pre-diabetic control when compared with the normal diet group without pre-diabetes [10, 11].

This study has shown that the IFG values for both the ND and HFHC-D fed groups fall just below the diagnostic range of pre-diabetes. However, literature has shown that defective insulin secretion and altered insulin sensitivity are already existing when fasting plasma glucose is still within the normal range, <5.6 mmol/L [33, 34]. Furthermore, its documented that the OGT and IFG tests detect insulin resistance only after it has progressed for a while and your blood sugar levels have started to rise [35]. Therefore, it is suggested that other tests for insulin resistance may be able to detect it earlier [35]. Therefore, despite the normal IFG and OGT levels, the utilization of glucose over the two-hour period (figure 1) do suggest the presence of moderate IR as the AUC value for the HFHC-D is higher than in the ND. Furthermore, the HFHC-D fed group failed to return glucose levels to its initial levels (IFG levels) meanwhile the ND fed group was able to return glucose levels to below its initial level.

The presence of moderate IR was further evidenced when we compared the pre-diabetes group/HFHC-D with the ND group as depicted in table 5, wherein the results show that a HFHC diet increased the plasma insulin levels and HOMA2-IR value. A HOMA2-IR value of >1,9 is regarded as a marker of early IR. A study in our laboratory displayed similar results as rats fed

with HFHC-D showed increased levels of plasma insulin and HOMA2-IR [10, 11]. According to literature, high insulin levels come about when the peripheral target tissues such as the skeletal muscle responds poorly to normal insulin concentrations, consequently the pancreatic β cell responds by producing more insulin to overcome the high blood glucose concentrations among IR peripheral target tissues [28, 36]. Consequences of high levels of insulin is strongly associated to metabolic complications such as heart attack and stroke [37].

The possible cause to moderate IR is multifactorial and increased lipids levels is one factor. The high levels of triglycerides in the blood of the HFHC-D fed group suggest that its' accumulation may be responsible for the development of moderate IR. This is because, high triglycerides levels in the blood may result in them accumulating in tissues such as adipose and skeletal muscle tissue. In literature, triglyceride accumulation in the muscle of high-fat diet-fed rats occur simultaneously with IR, which is affirms the hypothesis that IR is related to triglyceride accumulation in muscle [38]. Upon insulin stimulated glucose uptake into skeletal muscle cells, glucose is converted to glucose-6-phosphate by hexokinase II [39], in turn is metabolised via glycolysis, citric acid cycle and phosphorylation to form adenosine triphosphate (ATP) that is essential for cell metabolic processes [40]. Interestingly, the intermediates of the citric acid cycle are moved in the direction of lipid synthesis that is under insulin control in the muscle [41], which further explains the high triglyceride levels experienced in the HFHC-D group (table 8).

Additionally, after the formation of glucose-6-phospahte, a cascade of events lead to the synthesis of glycogen (see figure 3), a metabolic process which is also influenced by insulin signalling [42]. The findings of this study show increased glycogen concentrations in the HFHC-D fed group relative to ND group. This correlates with the high insulin levels observed in the HOMA2-IR index table, as insulin would promote glycogen synthesis. This finding is expected during the pre-

diabetes state as IR is still at moderate level and the pancreatic β cells haven't be exhausted as it would be seen in overt T2DM [28].

A gene is defined as a region of DNA or RNA that comprises a specific set of instructions, usually coding for a protein or for a function. During gene expression, the gene containing a nucleotide sequence determines the sequence of its mRNA product through a process of transcription. In turn, the mRNA sequence determines the amino acid sequence of the resulting polypeptide that make up a protein. Therefore, gene expression is a very critical state at which the genotype brings about the phenotype [43]. This is accomplished when the genetic code housed in the DNA is interpreted by gene expression, and the properties of the expression generates the organism's phenotype that is generally expressed through protein synthesis that act as functional enzymes catalyzing specific metabolic pathways [44]. Therefore, it can be assumed that the level of mRNA is directly proportional to its protein product.

The expression of GLUT-4 gene of interest expressed minimal change relative to the control, the reference gene. This could indicate that during the pre-diabetes state, the synthesis of the GLUT-4 protein remains normal. This could be explained by that during the prediabetic state, blood glucose levels remain below the diabetes thresholds [8], thus, the signal to upregulate GLUT-4 synthesis has not yet been triggered. However, the translocation of GLUT-4 to the plasma membrane in order to promote glucose uptake is dependent on the stimulation of active Akt [45]. The expression of Akt gene expresses a 4-fold increase in its expression. This increase may be interpreted as an increase in its protein expression. But, for Akt to be functional it requires an induction at PIP3 site in the presence of PDK1 [5]. Despite the increased synthesis of Akt protein suggested by the 4-fold increase in expression of its coded Akt gene, when taken together with the GLUT4 gene expression, the Akt results suggest that few of the Akt protein ended up being

activated as it would reflect by increased levels of GLUT 4. We suggest that there is partial disturbance in the insulin pathway probably because of the two-fold increase in the expression of P70S6 gene that codes for the protein P70S6. P70S6 stimulation is due to activated mTORC1 protein and its elevation is regarded a marker for insulin insensitivity because of its negative feedback signal on IRS [46]. This is achieved through stimulation of the serine-phosphorylation of IRS-1, which in turn disturbs the recruitment and triggering of PI3K resulting to reduced insulin activity [18, 47].

5.0 Conclusion

The presence of high HOMA2-IR index, triglycerides, glycogen and AUC value are indications of pre-diabetes. We suggest that the elevated triglyceride concentration observed in the HFHC-D induced prediabetic rats is a contributing factor for alterations in the insulin signaling pathway. We suggest that during pre-diabetes, moderate alterations in the insulin signaling pathway are already present, due to the current findings of a two-fold increase in gene expression of the P70S6, which is responsible for the disturbance of the insulin pathway at the IRS-1 resulting in IR.

6.0 Shortfalls and future studies

A shortfall of this study is the absence of IRS-1 gene expression that would have further validated the alteration of the insulin pathway at the IRS-1. Interestingly, however, gene expression is regulated at both the mRNA and protein level through on and off switches and fine-tuned control [43]. The alteration of gene expression can be achieved through prominent epigenetic mechanisms

such as DNA methylation, histone modification, and non-coding RNA-mediated pathways [48]. Therefore, in future studies, it would be interesting to see whether there is any presence of epigenetic changes in these genes of interest during the pre-diabetes stage. Also, in future, western blot analysis of the proteins involved in the insulin pathway can be conducted to further validate the current findings.

7.0 Acknowledgements

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8.0 List of references

1. Roglic G. WHO Global report on diabetes: A summary. International Journal of Noncommunicable Diseases. 2016;1(1):3.

2. Association AD. Diagnosis and classification of diabetes mellitus. Diabetes care. 2014;37(Supplement 1):S81-S90.

3. Schinner S, Scherbaum W, Bornstein S, Barthel A. Molecular mechanisms of insulin resistance. Diabetic Medicine. 2005;22(6):674-82.

4. Kahn S. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. Diabetologia. 2003;46(1):3-19.

5. Lv J, Yu C, Guo Y, Bian Z, Yang L, Chen Y, et al. Adherence to a healthy lifestyle and the risk of type 2 diabetes in Chinese adults. International journal of epidemiology. 2017;46(5):1410-20.

6. Kovacs P, Hanson RL, Lee Y-H, Yang X, Kobes S, Permana PA, et al. The Role of Insulin Receptor Substrate-1 Gene (IRS1) in Type 2 Diabetes in Pima Indians. Diabetes. 2003;52(12):3005-9.

7. Forouhi N, Luan J, Hennings S, Wareham N. Incidence of type 2 diabetes in England and its association with baseline impaired fasting glucose: the Ely study 1990–2000. Diabetic Medicine. 2007;24(2):200-7.

8. Tabák AG, Herder C, Rathmann W, Brunner EJ, Kivimäki M. Prediabetes: a high-risk state for diabetes development. The Lancet. 2012;379(9833):2279-90.

9. Mluleki Luvunyo MM, Andile Khathi. Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. Ponte Academic Journal 2017;74(5).

10. Mabuza LP, Gamede MW, Maikoo S, Booysen IN, Ngubane PS, Khathi A. Effects of a Ruthenium Schiff Base Complex on Glucose Homeostasis in Diet-Induced Pre-Diabetic Rats. Molecules (Basel, Switzerland). 2018;23(7):1721.

11. Gamede M, Mabuza L, Ngubane P, Khathi A. The Effects of Plant-Derived Oleanolic Acid on Selected Parameters of Glucose Homeostasis in a Diet-Induced Pre-Diabetic Rat Model. Molecules. 2018;23(4):794.

12. Mukundwa A, Mukaratirwa S, Masola B. Effects of oleanolic acid on the insulin signaling pathway in skeletal muscle of streptozotocin-induced diabetic male Sprague-Dawley rats. Journal of diabetes. 2016;8(1):98-108.

13. Barrett K, Barman S, Boitano S, Brooks H. Ganong's review of medical physiology. Chapter 21. Endocrine functions of the pancreas & regulation of carbohydrate metabolism. 23 ed2010. 727 p.

14. Bouché C, Serdy S, Kahn CR, Goldfine AB. The Cellular Fate of Glucose and Its Relevance in Type 2 Diabetes. Endocrine Reviews. 2004;25(5):807-30.

15. Peti W, Page R. Molecular basis of MAP kinase regulation. Protein science. 2013;22(12):1698-710.

16. Takei N, Nawa H. mTOR signaling and its roles in normal and abnormal brain development. Frontiers in molecular neuroscience. 2014;7:28.

17. Vergès B, Cariou B. mTOR inhibitors and diabetes. Diabetes research and clinical practice. 2015;110(2):101-8.

18. Blagosklonny M. TOR-centric view on insulin resistance and diabetic complications: perspective for endocrinologists and gerontologists. Cell death & disease. 2013;4(12):e964.

19. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology. 2011;12(1):21.

20. Ngubane PS, Masola B, Musabayane CT. The effects of Syzygium aromaticum-derived oleanolic acid on glycogenic enzymes in streptozotocin-induced diabetic rats. Renal Failure. 2011;33(4):434-9.

21. Ong KC, Khoo H-E. Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. Life sciences. 2000;67(14):1695-705.

22. Geloneze B, Vasques ACJ, Stabe CFC, Pareja JC, Rosado LEFPdL, Queiroz ECd, et al. HOMA1-IR and HOMA2-IR indexes in identifying insulin resistance and metabolic syndrome:

Brazilian Metabolic Syndrome Study (BRAMS). Arquivos Brasileiros de Endocrinologia & Metabologia. 2009;53:281-7.

23. Levy JC, Matthews DR, Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. Diabetes care. 1998;21(12):2191-2.

24. Anier K, Malinovskaja K, Aonurm-Helm A, Zharkovsky A, Kalda A. DNA methylation regulates cocaine-induced behavioral sensitization in mice. Neuropsychopharmacology. 2010;35(12):2450.

25. Karpovets T, Konopelnyuk V, Galenova T, Savchuk A, Ostapchenko L. High-calorie diet as a factor of prediabetes development in rats. Bulletin of experimental biology and medicine. 2014;156(5):639.

26. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. The American journal of clinical nutrition. 2005;81(2):341-54.

27. Mayans L. Metabolic Syndrome: Insulin Resistance and Prediabetes. FP essentials. 2015;435:11-6.

28. Wang G. Raison d'être of insulin resistance: the adjustable threshold hypothesis. Journal of the Royal Society, Interface. 2014;11(101):20140892-.

29. Zimmet P, Alberti K, Shaw J. Global and societal implications of the diabetes epidemic. Nature. 2001;414(6865):782.

30. Bartoli E, Fra G, Schianca GC. The oral glucose tolerance test (OGTT) revisited. European journal of internal medicine. 2011;22(1):8-12.

31. Balion CM, Raina PS, Gerstein HC, Santaguida PL, Morrison KM, Booker L, et al. Reproducibility of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) classification: a systematic review. Clinical Chemical Laboratory Medicine. 2007;45(9):1180-5.

32. Libman I, Barinas-Mitchell E, Bartucci A, Robertson R, Arslanian S. Reproducibility of the oral glucose tolerance test in overweight children. The Journal of Clinical Endocrinology & Metabolism. 2008;93(11):4231-7.

33. Godsland I, Jeffs J, Johnston D. Loss of beta cell function as fasting glucose increases in the non-diabetic range. Diabetologia. 2004;47(7):1157-66.

34. Gastaldelli A, Ferrannini E, Miyazaki Y, Matsuda M, DeFronzo R. Beta-cell dysfunction and glucose intolerance: results from the San Antonio metabolism (SAM) study. Diabetologia. 2004;47(1):31-9.

35. Cobb J, Gall W, Adam K-P, Nakhle P, Button E, Hathorn J, et al. A novel fasting blood test for insulin resistance and prediabetes. Journal of diabetes science and technology. 2013;7(1):100-10.

36. Gill H, Mugo M, Whaley-Connell A, Stump C, Sowers JR. The key role of insulin resistance in the cardiometabolic syndrome. The American journal of the medical sciences. 2005;330(6):290-4.

37. Lakka H-M, Lakka TA, Tuomilehto J, Sivenius J, Salonen JT. Hyperinsulinemia and the risk of cardiovascular death and acute coronary and cerebrovascular events in men: the Kuopio Ischaemic Heart Disease Risk Factor Study. Archives of Internal Medicine. 2000;160(8):1160-8.

38. Turner N, Cooney GJ, Kraegen EW, Bruce CR. Fatty acid metabolism, energy expenditure and insulin resistance in muscle. Journal of Endocrinology. 2014;220(2):T61-T79.

39. Lehto M, Xiang K, Stoffel M, Espinosa R, Groop LC, Le Beau MM, et al. Human hexokinase II: localization of the polymorphic gene to chromosome 2. Diabetologia. 1993;36(12):1299-302.

40. Guo X, Li H, Xu H, Woo S, Dong H, Lu F, et al. Glycolysis in the control of blood glucose homeostasis. Acta Pharmaceutica Sinica B. 2012;2(4):358-67.

41. Nuttall FQ, Ngo A, Gannon MC. Regulation of hepatic glucose production and the role of gluconeogenesis in humans: is the rate of gluconeogenesis constant? Diabetes/metabolism research and reviews. 2008;24(6):438-58.

42. Adeva-Andany MM, González-Lucán M, Donapetry-García C, Fernández-Fernández C, Ameneiros-Rodríguez E. Glycogen metabolism in humans. BBA clinical. 2016;5:85-100.

43. Zhang X, Ho S-M. Epigenetics meets endocrinology. Journal of molecular endocrinology. 2011;46(1):R11-R32.

44. Liu Y, Beyer A, Aebersold R. On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell. 2016;165(3):535-50.

45. Lee J, Pilch P. The insulin receptor: structure, function, and signaling. American Journal of Physiology-Cell Physiology. 1994;266(2):C319-C34.

46. Stanley M, Macauley SL, Holtzman DM. Changes in insulin and insulin signaling in Alzheimer's disease: cause or consequence? The Journal of Experimental Medicine. 2016;213(8):1375-85.

47. Rivas DA, Yaspelkis BB, Hawley JA, Lessard SJ. Lipid-induced mTOR activation in rat skeletal muscle reversed by exercise and 5′-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside. Journal of Endocrinology. 2009;202(3):441-51.

48. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell. 2007;128(4):669-81.

Chapter 3 (Synthesis)

Alterations of insulin signalling pathway during the prediabetic state

Pre-diabetes is a previously disregarded condition that generally propagates the development of T2DM when left unattended. The characterization of pre-diabetes includes the presence of moderate hyperglycaemia and insulin resistance (IR). The fact that some diabetic complications are observed 4-7 years prior a person is diagnosed with T2DM is a reason for concern. Thus, because pre-diabetes often precedes T2DM, more attention should be given to pre-diabetes so that more awareness is relayed not only amongst researchers but even to the general population.

In our laboratory, a diet that is rich in fats and carbohydrates was able to induce a pre-diabetes Sprague Dawley rat model. Additionally, this was achieved along with weight gain/obesity, which is associated with metabolic complications such as pre-diabetes. However, the mechanistic leading to IR hasn't been investigated in this model. Therefore, this study aims to investigate the changes in the insulin signalling pathway in diet-induced pre-diabetic Sprague Dawley rats. Therefore, the same high fat high carbohydrates diet (HFHC-D) was used in this study to induce pre-diabetes in Sprague Dawley rats for a period of 20 weeks. The OGT test was performed along with triglyceride concentrations prior the termination of the animal work. Thereafter biochemical analyses such as the ELISA, glycogen assay and PCR were performed to achieve the aim of the study.

The findings show that a HFHC-D was able to induce pre-diabetes over the induction period of >20 weeks. This was supported by the higher AUC of the OGT test graph, higher plasma triglycerides and higher HOMA2-IR index in the HFHC-D group compared to the ND/Control

group. The HFHC-D fed group failed to return glucose levels to its initial levels (IFG levels) meanwhile the ND fed group was able to return glucose levels to below its initial level. This is as an indication of a poor response to the hormone insulin in the skeletal muscle tissue of the HFHC-D group, because an ideal response to insulin would be to restore glucose levels back to its initial state following the oral dosage of glucose. These results suggest the presence of pre-diabetes in these rats. Following this prediabetic induction process, the next step was to assess the state of the insulin signalling pathway by analysis of gene expression of genes coded for proteins involved in the insulin pathway and the expression of these proteins. Real time PCR was used to measure gene expression and the Western blot technique was used to measure protein expression.

The findings of the gene expression of Akt, GLUT-4 and p70S6 altogether do suggest that there are changes in the insulin pathway although still at a moderate level. The two-fold increase of the gene p70S6 that is responsible inhibiting the insulin pathway at the IRS-1 is an indication of impaired insulin pathway. Also, physiologically, the increase of the Akt protein is expected to be in proportion with the increase of the GLUT-4 protein. However, the PCR results show an inversely proportional relationship between Akt, and GLUT-4 as seen with the 4-fold increase in the Akt gene expression and normal GLUT-4 expression when there are compared to the ND/Control group. This is interpreted as that there is a poor activation of the Akt that leads to the translocation of GLUT-4 to the plasma membrane. Thus, further suggesting an impairment in the signalling pathway in these prediabetic rats. However, the insulin signalling pathway consists of a lot of other proteins that regulate other functions therefore more work is still required to elucidate the effects of this diet on their expression.

List of appendixes

Appendix 1: Ethical clearance

18 July 2017

Mr Aubrey Sosibo (213514382)
School of Laboratory Medicine and Medical Sciences Westville Campus

Dear Mr Sosibo,

Protocol reference number: AREC/026/017M
Project title: The effects of high-fat high-carbohydrate diet on the expression of mTOR in Sprague-Dawley rats

Full Approval — Research Application With regards to your revised application received on 04 July 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

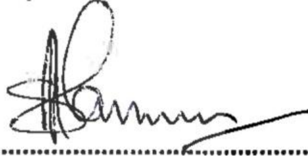
Any alteration/s to the approved research protocol, i.e. Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 18 July 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.
Yours faithfully



Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Dr Andile Khathi
Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Stephanie Keulder
cc BRU - Dr Sanil Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
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Website: hNp://research. ukzn.ac.za/ResearGh-Ethics/Animal-Ethics.aspx

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Appendix 2: Travel award

Appendix 3: Manuscript Organization (PLOS ONE)

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* • Title page: List title, authors, and affiliations as first page of manuscript • Abstract • Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* • Materials and Methods • Results • Discussion • Conclusions (optional)
Ending section	*The following elements are required, in order:* • Acknowledgments • References • Supporting information captions (if applicable)
Other elements	• Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately.

	• Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.

Appendix 4: Conference presentation

Abstract title	Event	Category	Presentation	Status	Action required
The effects of diet-induced Pre-diabetes on selected markers of glucose homeostasis in male Sprague Dawley rats	CoBNeST 2018	PSSA	Poster	Abstract accepted	-