THE CHANGES IN IMMUNE CELLS CONCENTRATION DURING THE PROGRESSION OF PRE-DIABETES TO TYPE 2 DIABETES IN A DIET-INDUCED PRE-DIABETIC RAT MODEL.

By

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PREFACE

The continuous consumption of high-fat high-carbohydrate (HFHC) diet with sedentary lifestyle has been shown to cause type 2 diabetes (T2D) in human beings. However, human beings suffer from pre-diabetes for a long period before onset of T2D. It has also been shown that type 2 diabetic patients have suppressed immune system due to dysregulated innate immunity. However, it's still unclear whether the immune activation occurs at a pre-diabetic stage. A rat model has been discovered that mimics the western or high-fat high-carbohydrates consumed by humans with sedentary lifestyle for detailed research based on the pre-diabetic stage that precedes T2D. In this study, the aim is to characterise if there are changes in immune cell concentration during the progression of pre-diabetes stage and evaluate if immune activation occurs at pre-diabetes stage. The experimental work described in this dissertation was conducted at University of Kwa-Zulu natal, Westville campus, Durban, South Africa. Lipid profile samples we analysed at Global Clinical and Viral laboratory at Amanzimtoti, Durban, South Africa. It was conducted under supervision of Dr Andile Khathi and co-supervised by Dr Phikelelani Ngubane.

DECLARATION

I, Nomusa Christina Mzimela hereby declare that the dissertation entitled:

"The changes in immune cells concentration during the progression of pre-diabetes to type 2 diabetes in a diet-induced pre-diabetes rat model" 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.

Student: N.C Mzimela Signature _____Date: _____

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DEDICATION

This work is dedicated to my parents. I can imagine the smile in your faces. I know you are both happy and proud.

ACKNOWLEDGEMENTS

To God be the glory. I would like to express my very profound gratitude to my family for believing in me, continuous encouragements and unfailing support. Ngithi ngiyabonga boNjingili, Mnguni, Lulwandle aluwelwa, Luwelwa zinkonjane zona ezindizela phezulu, Zimeme, Maconi, Donda kaMlimandlela, Ntaka, Mfeka, Sheleza, Mzungwana, Mwelase, Ongaweli ngezibuko, Owela ngezimpambose zomfula…

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LIST OF ABBREVIATIONS

Abbreviation	Name
ADP	Adenosine diphosphate.
AGEs	Advanced glycation end-products.
AP-1	Activator protein-1.
BRU	Biomedical Resource Unit.
CD40L	Cluster of differentiation 40 ligand.
CRP	C-reactive protein.
DAG	Diacylglycerol.
DPX	Distyrene Plasticizer Xylene.
EDTA	Ethylene diamine tetra acetic acid.
ELISA	Enzyme linked immunosorbent essay.
FFAs	Free fatty acids.
FPA	Fibrinopeptide A.
FPB	Fibrinopeptide B.
HbA1c	Glycated haemoglobin.
HCD	High-carbohydrate diet.
HDL	High density lipoprotein.
HFD	High-fat diet.

HFHC	High-fat high-carbohydrate diet.
IGT	Impaired glucose tolerance.
IKKB	Inhibitor kappa B kinase.
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-6	Interleukin-6
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
JNK	C-Jun N-terminal kinase.
LDL	Low Density Lipoprotein.
LFD	Low-fat diet.
MAP	Mean arterial pressure.
MAPK	Mitogen-activator protein kinase.
MCP-1	Monocyte chemoattractant protein-1
mTOR	Rapamycin
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase.
NC	Non-diabetic control.
NF-κB	Nuclear factor kappa B.
NHANES	National Health and Nutrition Examination Survey.

NO	Nitric oxide
NRF	National Research Foundation
NS	Nitric oxide species.
OGTT	Oral glucose tolerance test.
ox LDL	Oxidised low-density lipoprotein.
PAI-1	Plasminogen activator inhibitor-1
PD	Pre-diabetic group
PI3K	Phosphoinositide 3 kinase.
PKC	Protein kinase C.
PSGL-1	P-selectin glycoprotein ligand-1
RAGEs	Advanced glycation end-product receptor.
ROS	Reactive oxygen species.
SFAs	Saturated fatty acids.
T2D	Type 2 diabetes
TG	Triglyceride
TLR 2	Toll like receptor 2
TLR 4	Toll like receptor 4
TNF-A	Tumour necrosis factor-alpha.
UKZN	University of Kwa-Zulu Natal.

VCAM-1	Vascular adhesion molecule-1
vWF	von Willebrand factor.
WHO	World Health Organisation.

LIST OF APPENDICES

Presentations

Symposium presentation.

Mzimela, N.C, Ngubane, P.S, Khathi, A**.** The changes in immune cells concentration during the progression of pre-diabetes to type 2 diabetes in a diet-induced pre-diabetes rat model. School of Laboratory Medicine and Medical Sciences Symposium,29 August 2018, University of Kwa-Zulu Natal, Durban, South Africa.

Conference presentation.

Mzimela, N.C, Ngubane, P.S, Khathi, A. The changes in immune cells concentration during the progression of pre-diabetes to type 2 diabetes in a diet-induced pre-diabetes rat model. 1st Conference of Biomedical and Natural Sciences and Therapeutics (CoBNEST), 7-10 October 2018, Spier conference centre, Stellenbosch, Cape Town, South Africa.

STUDY OUTLINE

The current dissertation is presented in manuscript format, consisting of 6 sections viz. chapter 1: introduction /literature review, chapter 2: prologue, abstract and manuscript 1, chapter 3: synthesis and appendices. Chapter 1 lay a foundation of the literature and the information that has been discovered in order to link the area of interest of the study and cover a gab that is a debatable issue. Chapter 2 contains a prologue of the research of interest, the abstract of the study and the manuscript of the research that has covered a gab of the immune cell concentration changes during progression of pre-diabetes to type 2 diabetes and upregulation of inflammatory markers such as IL-6, TNF-α, CRP, fibrinogen, P-selectin and CD40L at pre-diabetes stage. This is the work authored by A Khathi, P.S Ngubane and N.C Mzimela. This work has been published in **Autoimmunity** (Impact factor 2.648). Chapter 3 discusses the synthesis of the study and the appendix consists of the journal's guideline to authors, conference attendance and ethics certificates.

ABSTRACT

T2D has been discovered to be preceded by a long-lasting condition known as prediabetes. The primary cause of prediabetes and T2D has also been shown to be continuous consumption of unhealthy diets and living a sedentary lifestyle. Type 2 diabetic patients have been discovered to have a supressed immune system, but it is still debatable whether immune activation begins at the pre-diabetes stage or during overt T2D. According to literature, T2D is a result of elevated levels of glucose known as hyperglycaemia caused by a condition called insulin resistance. Additionally, T2D has also been shown to be characterised by increased levels of triglycerides, low density lipoproteins (LDL) and decreased levels of high-density lipoproteins (HDL). Insulin resistance then causes metabolic and signalling pathways such as oxidative stress, activation of PKC pathway, formation of advanced glycation end products (AGEs) and shunting of polyol pathway which trigger metabolic inflammation resulting in a dysregulated innate immunity. Dysregulated innate immunity in T2D patients has also been discovered to be due immune response caused by hyperglycaemia. However, it has not been discovered if immune activation occurs at the pre-diabetes stage. It has not been discovered if upregulation of inflammatory markers occurs at prediabetes stage. This study envisaged to characterise the changes that occur in immune cell concentration during the progression of pre-diabetic stage and if there is upregulation of inflammatory markers such as fibrinogen, CRP, CD40L, p-selectin, IL-6 and TNF-α during pre-diabetic stage. To accomplish this, male Sprague Dawley rats were divided into two groups. The first group was fed a high-fat high-carbohydrate diet for 20 weeks to induce pre-diabetes and the second group was fed a normal rat diet for 20 weeks. To confirm if the animals were pre-diabetic, criteria according to American Diabetes Association were used. The animals were then divided into 2 groups which is the pre-diabetic group with 6 animals and a non-diabetic control with another 6 animals. The animals were then further monitored for another 12 weeks (experimental period) while fed the same diet. Blood

was collected for haemocytometer analysis on week 0,4,8 and 12 of the experimental periods after which the animals were sacrificed. Plasma was collected from centrifuged blood for ELISA (TNF- α, CRP, P-selectin, CD40 L, fibrinogen & IL-6). Adipose tissue was collected for histology. The results showed a significant decrease in blood percentage count of neutrophils and eosinophils at week 12 experimental period and these immune cells were further observed embedded in-between the adipocytes of adipose tissue. This indicated that neutrophils and eosinophils are produced due to hyperglycaemia and then recruited to the inflamed area such as adipose tissue. The blood percentage count of lymphocytes, basophils and monocytes showed a significant increase at week 12, indicating their increase in production in the bone marrow during immune response. Additionally, the results showed a significant increase in inflammatory cytokines such as TNF-α, IL-6, CRP and P-selectin. The results also showed a slight increase in inflammatory markers such as CD40L and fibrinogen. These finding indicate that there is immune activation during pre-diabetes stage due to changes in immune cells concentration and upregulation of inflammatory markers.

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Type 2 diabetes (T2D) is characterised by hyperglycaemia which contributes to metabolic and signalling abnormalities such as increased oxidative stress, activation of polyol pathway, activation of protein kinase C (PKC) pathway and formation of advanced glycation end products (1). However, the onset of T2D is often preceded by a long lasting condition called pre-diabetes (2). It still remains unclear if these metabolic and signalling abnormalities begin during the pre-diabetic stage. Kayal and Graves discovered that in T2D, hyperglycaemia causes increases in oxidative stress, shunting of polyol pathway ,activation of PKC pathway and formation of advanced glycation end products (1). Furthermore, Kayal and Graves discovered these abnormalities lead to a dysregulated innate immunity system (1). According to literature, in T2D, during an immune response caused by hyperglycaemia there is increased production of immune cells such as neutrophils, basophils, lymphocytes, monocytes and eosinophils (3-10). It has also been discovered that tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are elevated in T2D (11, 12). Furthermore, innate immunity dysregulation and chronic secretion of pro-inflammatory cytokines contribute to type 2 diabetes complications (1, 6, 13-15). The signalling and metabolic abnormalities caused by hyperglycaemia in T2D result in hyper-reactive platelets (16). According to literature, T2D have elevated levels of P-selectin, soluble cluster of differentiation 40 ligand (CD40L) and C-reactive protein (CRP) which cause accumulation of platelets in vessel walls (17, 18). However, it also remains unclear if these changes occur during the pre-diabetes stage. One of the complications associated with T2D is atherosclerosis which leads to cardiovascular complications (19). Atherosclerosis is the hallmark of cardiovascular diseases characterised by hyperfibrinogenaemia due to clot formation, increased oxidised low-density lipoproteins (ox LDL) as well as decreased bioavailability of nitric oxide (NO) due to endothelial damage (20-22). The endothelial damage

is caused by oxidative stress in T2D (23). However, it still remains unclear if pre-diabetics are at risk of developing atherosclerosis. Therefore, this study sought to evaluate the state of general immunity during the progression of pre-diabetes and accumulation of platelets in vessel wall at the pre-diabetes stage.

1.2. State of immunity in T2D

T2D is associated with insulin resistance results in hyperlipidaemia, hypertension which triggers the immune response (24, 25). According to Sindhu *et al*, in obese individuals and rodents, the chronic low grade systemic inflammation called metabolic inflammation has been to be a contributing factor associated with induction of insulin resistance and development of T2D (26). In obese subjects, metabolic inflammation with oxidative stress caused by the imbalance between free radicals and anti-oxidants has also been implicated to increase the progression of insulin resistance and development of T2D (27). However, due to chronic hyperglycaemia, the immune response is also said to be triggered by glucotoxicity (25). During the immune response there is increased production of immune cells such as neutrophils, monocytes, lymphocytes, eosinophils and basophils in the bone marrow (6, 8, 9, 28). These recruited immune cells play different roles in the immune system and contribute to inflammation and progression of type 2 diabetes (6, 8, 9).

1.2.1. Neutrophils

During hyperglycaemic conditions, the production of neutrophils is increased and according to literature they are the first immune cells to migrate from circulation to the inflamed area for immune response where they secrete cytokines such as TNF-α, interleukin-1 (IL-1) and IL-6 (5). Furthermore, hyperglycaemia causes gradual formation and accumulation of neutrophil extracellular traps (5). This then results in neutrophils eliminating pathogens as an active constituent of innate immune system producing basal levels of IL-6 due to glucotoxicity (5).

According to Papayannopoulos, due hyperglycaemia in T2D, ROS generated by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) activates neutrophils resulting in activation of PKC (29). Additionally, Hatanaka *et al* discovered that the rise in intracellular Ca^{2+} of neutrophils in T2D which is triggered by hyperglycaemia and presence of AGEs stimulate cytokine release and accumulation, for inflammation in the inflamed areas (30). The accumulated and released cytokines activate phagocytes to release lytic proteins for phagocytosis (30, 31). The cytokines that are released by these chronically activated phagocytes then trigger apoptosis and cause more formation of ROS which further cause more inflammation contributing to progression of T2D (29). Cytokines secreted by neutrophils such as IL-6,IL-1β,TNF-α and IL-8 are responsible for inflammation, contributing to progression of T2D (5, 9). However, it remains unclear if these characteristics occur during the pre-diabetes stage.

1.2.2. Lymphocytes

Due to glucotoxicity caused by hyperglycaemia, there is increased production of lymphocytes in the bone marrow resulting in T-cells secreting the cytokines such as IL-6 for inflammation (8, 9). B-cells recruited mature into plasma cells and then secrete antibodies such as IgE, IgM. B-cells also secrete cytokines such as IL-6 during immune response for inflammation (4, 32). According to Libby, B and T lymphocytes has also been shown to be one of the immune cells that join the macrophages in the intima during the lesion evolution in the atherosclerosis, which is one of the T2D complications (33). This contributes to the progression of T2D. However, no studies have been done based on these characteristics during the pre-diabetes stage.

1.2.3. Basophils

The recruited basophils display the IgE antibody receptor on their surface (4, 34). Moreover, once they are recruited, they bind to IgE antibodies secreted by B-cells to activate them since high-carbohydrate diet is associated with increased IgE serum, causing release of cytokines

such as IL-6, VCAM-1 and IL-4 for inflammation (4, 32). Once the basophils are activated, they regulate eosinophil accumulation by activating a vascular cell adhesion molecule (VCAM-1) which is required for the recruitment of eosinophils (8). According to Libby, VCAM-1 has also been observed to be able to bind precisely the types of leukocytes such as monocytes and T-lymphocytes in the early human and experimental atheroma during development of atherosclerosis (33). This contribute to the progression of T2D. However, it still a debatable issue if these characteristics occur at the pre-diabetes stage

1.2.4. Eosinophils

In T2D, the produced and circulating eosinophils has been shown to migrate to the inflamed area where they secrete cytokines such as IL-4 to sustain alternatively activated macrophages for inflammation which contribute to progression of T2D (35). According to Che *et al*, eosinophils are also required for the maintenance of plasma cells in the bone marrow (3). Secretion of cytokines by immune cells then result in the elevated levels of TNF-α and IL-6 for inflammation contributing to progression of T2D. However, the characteristics of eosinophils at pre-diabetes stage has never been discovered.

1.2.5. *Monocytes*

Increased production of monocytes results in monocytes secreting cytokines upon activation during immune response (9). However, in the inflamed area the macrophages secrete cytokines such as monocyte chemoattractant protein-1 (MCP-1) due to inflammation (34). MCP-1 expression then results in recruitment of monocytes to the inflamed area where they differentiate into macrophages secreting more cytokines for inflammation (10, 28, 36, 37). Due to hyperglycaemia, more monocytes are produced in the bone marrow resulting in monocytes taking up glucose to decrease their concentration to normal from circulation (38). Monocytes have been discovered to have glucose transporters allowing the uptake of glucose (38).

Furthermore, monocytes also respond to high carbohydrate diet by NADPH oxidase mediating high glucose induced nuclear factor kappa B (NF-$_k$B) activation and releasing enhanced IL-6 and IL-1β expression (10, 36, 39). NF-$_k$B is also bought into play also by the macrophage contact with the medium from adipocytes resulting in release of cytokines for inflammation (40). Additionally, in T2D, monocytes have been shown to migrate to the intima where they differentiate to macrophages (41). Moreover these macrophages accumulate LDL and differentiate into foam cells in atherosclerosis (42, 43). These characteristics of monocytes contribute to the progression of T2D and progression of atherosclerosis in T2D. However, it remains unclear if these characteristics occur at pre-diabetic stage. Due to changes in immune cell concentration, formation of advanced glycation end product and increased oxidative stress, T2D patients are at high risk of atherosclerosis due to endothelial damage (19, 22, 44-46).

1.3. Increase in oxidative stress cause damage to endothelial cells

According to Pitocco *et al*, the cardiovascular risk factors in diabetic conditions has been shown be induced by ROS production in the endothelial cells (22). Specifically, according to Kaneto *et al,* its hyperglycaemia that has been shown to induce ROS through activation of glycation reaction and electron transport chain in the mitochondria (47). Additionally, AGEs, insulin and angiotensin II have also been shown to induce ROS through activation of the NADPH oxidase (47). According to Basta *et al,* AGEs in vasculature interact with the endothelial surface RAGE to induce intracellular ROS thereby activating the NADPH oxidase system (44). NADPH oxidase is the major source of ROS in vasculature (44). This production of ROS inactivates eNOS resulting in decreased NO levels in the endothelium which enable platelet adhesion and aggregation since NO inhibit platelet aggregation (44). Additionally, this production of ROS activates the NF-$_k$ B transcription factors which are highly relevant to inflammation, immunity and atherosclerosis (48).

Moreover, hyperglycaemia causes oxidative stress due to overproduction of reactive oxygen species (ROS), nitric oxide species (NS) and potent radicals such as hydrogen peroxide and superoxide anions (20, 22). This then causes damage to the endothelium and reduces the production of nitric oxide and prostacyclin which are potent vasodilators responsible for inhibiting platelet aggregation (49). Hypercholesterolemia is one of the characteristics of T2D (42).

In our laboratory, we have shown that during the pre-diabetic stage the animals display increased LDL and decreased HDL levels (50). According to literature increased levels of LDL in T2D due to hypercholesterolemia result in LDL binding to the free radicals in the endothelium which then impair the endothelial nitric oxide synthase (eNOS) (42). Furthermore, nitric oxide can be oxidised by the superoxide ions due to oxidative stress leading to further reduction in its half-life and anti-platelet action (22). These circulating LDL bind to the free radicals in the endothelial cells to become oxidised LDL (oxLDL) (42). According to literature ox LDL impair endothelial nitric oxide synthase which then result in the decreased bioavailability of nitric oxide in the endothelial cells (42). Nitric oxide is a vasodilator for endothelial cells and also inhibit adhesion, aggregation and coagulation of platelets in the endothelial cells (22). Therefore, the absence of vasodilators causes vasoconstriction and promotes the accumulation of platelets in the damaged endothelium (22, 33, 51, 52). Increase in oxidative stress cause damage to the endothelial cells resulting in the denudation of the endothelium (53, 54).

At the site of the vascular lesion, the extracellular matrix is exposed to blood stream thereby exposing the collagen and von Willebrand Factor (vWF) (54). Expression of vWF by damaged endothelium cause the recruitment of platelets to the endothelial wall (54). Collagen binds to glycoprotein Ia/IIa expressed by platelets and vWF bind to glycoprotein Ib/IX also expressed by platelets (54). According to literature, this then triggers the platelets to release the content

of their secretory vesicles, which contains variety of chemical agents such as adenosine diphosphate (ADP), thromboxane A_2 and serotonin which act locally by inducing the change in metabolism, shape and surface proteins of platelets by process called activation of platelets (54, 55). Binding of collagen and vWF to these proteins also results in activation of integrin $\alpha_{11b}\beta_3$ (GP11a/111b) which is a fibrinogen receptor for binding of fibrinogen (56). Furthermore, AGEs formed due to hyperglycaemia bind the advanced glycation end products receptor (RAGEs) to activate the circulating platelets (44).

Upon activation, the platelet GP11a/111b is activated resulting in exposure to fibrinogen binding site (55, 57). This enables the recruitment of activated platelets to adhere to the adhered platelets that has already adhered to endothelial wall through binding to fibrinogen (57). This then forms the fibrinogen bridges linking platelets causing aggregation of platelets therefore accumulating in a vessel wall forming a platelet plug (57, 58).

Moreover, while a plug is built up and compacted, the vascular smooth muscle in the damaged vessel is then being stimulated to contract (58). This then decreases the blood flow in the area and change pressure in the vessel resulting in vasoconstriction (33).

This vasoconstriction then results in a cascade of enzyme activations which requires platelets, plasma cofactors and calcium anions causing conversion of plasma protein prothrombin to enzyme thrombin (33, 59). Moreover, this thrombin then catalyses the reaction in which several polypeptides are split from molecules of large rod-shaped protein fibrinogen (59, 60). However, the fibrinogen remnants then bind to each other and then form fibrin which its chemical linking is enzymatically catalysed by factor XIIIa. Factor XIII is formed from plasma protein factor XIII catalysed by thrombin which stabilises the fibrin meshwork (59, 60). This result in a formation of a clot (59, 60).This formation of the clot is the foundation of development of cardiovascular complications in T2D due to atherosclerosis (19, 59). However, it remains

unclear if these characteristics occur at a pre-diabetes stage. Additionally, cytokines have been shown to be a link between the immune cells and inflammation (61). Cytokines such as IL-6 and TNF-α influences immunity, inflammation and contribute to progression of T2D (11, 62, 63).

1.4. Inflammatory Markers

1.4.1. TNF-α and IL-6 as markers of immunity in T2D

It has been discovered that IL-6 and TNF-α are elevated in type 2 diabetes and they also contribute to progression of type 2 diabetes (12, 64-66). Interestingly, according to Pickup, these two cytokines have many pro-atherosclerotic actions including promotion of leukocyte recruitment to the endothelium by inducing the adhesion molecules and the synthesis of the chemoattractant and also raising the capillary permeability (61, 67, 68). These cytokines are also produced by the endothelium, smooth muscle cells and macrophages at the site of atherosclerosis (69). Furthermore, these two cytokines play a number of other roles contributing to changes in immune system, inflammation and progression of type 2 diabetes (61, 70, 71).

Furthermore IL-6 has been discovered to be a pleiotropic cytokine (62, 65). According to literature this cytokine display pro and anti-inflammatory effects (65). It is secreted by a variety of tissues and cells which can be activated leukocytes, adipocytes, macrophages and endothelial cells (37, 72). Interestingly, IL-6 induces gluconeogenesis, hyperinsulinemia and hyperglycaemia which end up causing metabolic syndrome and then overt type 2 diabetes (72-74). Monocytes play a central role in innate immunity by increasing secretion of IL-6 due to hyperglycaemia (39). However acute hyperglycaemia caused by insulin resistance due to high carbohydrate diet induces IL-6, TNF-α and IL-8 concentration (39, 75). Adiposity is also directly correlated with circulating levels of IL-6 and TNF-α (75). Exposure to chronic hyperglycaemic conditions also increase IL-6 secretion in 3T3-LI adipocytes(71, 76).

Moreover, in adipocytes, lipids induce endoplasmic reticulum stress by increasing JNK and extracellular receptor (77).

Furthermore , high fat diets activate protein kinase C, NF-$_k$ B and mitogen-activated protein kinase (MAPK) signalling which also contribute to cytokines production in 3T3-LI adipocytes (27). IL-6 inhibits lipoprotein lipase and stimulate lipolysis which then induce insulin resistance contributing to progression of type 2 diabetes (78). IL-6 secretion due to high fat diets also act upon insulin receptor substrate-1 when insulin is chronically elevated which then results in insulin availability phosphorylating IRS-1 on the serine residue to down regulate the phosphoinositide 3 kinase (PI3-K) (48, 79). This occurs via activation of mTor (rapamycin) which is activated by Akt (79). These 2 pathways activates protein kinase C signalling and nuclear factor kappa B pathway further cause chronic hyperglycaemia contributing to inflammation and progression of type 2 diabetes (79). However, the characteristic of IL-6 remains unclear at pre-diabetes stage.

Another cytokine elevated in type 2 diabetics is the monokine cytokine from the TNF family known as TNF-α (51). TNF-α is produced by macrophages and cells such as fibroblast, epithelial cells, adipocytes and myocytes (80). According to literature, this cytokine plays a role in the pathogenesis of insulin resistance since it is found elevated in adipocytes and plasma of insulin resistant subjects (66, 81). Furthermore, hyperglycaemia and hyperinsulinemia has been shown to increase the production of TNF-α from monocytes and macrophages *in vitro* (36, 74). Moreover, chronic hyperglycaemia activates macrophages and then stimulate TNF-α production (66). This increase the permeability of endothelium through release of nitric oxide by nitric oxide synthase which lead to depression of the myocardial myocytes (33, 51). This increase in TNF-α production also cause increase thrombogenesis through expression of plasminogen activator inhibitor -1 (PAI-1) and stimulate the expression of adhesion molecules on the endothelial cell (51, 55)

Increase in TNF-α production also induces apoptosis in myocytes and endothelial cells which then contribute to heart failure through apoptosis (52, 82). This TNF-α production also activates the protein kinase C signalling and nuclear factor kappa B signalling (48, 79). Therefore, increased TNF-α production contribute to type 2 diabetics complications during progression of type 2 diabetes (48, 79). However, the state of TNF-α at pre-diabetes stage is still a debatable issue and has never been discovered. Furthermore, there are markers such as CRP and fibrinogen that have been shown to be elevated in T2D.

1.4.2. C-reactive protein and fibrinogen as markers for events in atherosclerosis

According to literature, CRP is from a pentraxin family of innate immune response proteins (18). According to Frostegard, the increased levels of c-reactive protein (CRP) have been implicated as a risk marker for atherosclerosis and the developed cardiovascular diseases in many studies (83-85). Furthermore, according to literature, CRP is an acute phase reactant of hepatic origin that is synthesized largely under the influence of IL-6(18, 86). CRP has also been discovered to be a mediator for atherogenesis by quenching nitric oxide (19). According to Badimon and Vilahur, CRP has been shown to exert a thrombotic activity (87). CRP monomeric form plays a role in platelet adhesion by initiating platelet deposition and also a thrombus growth (87). Moreover CRP dissociation from its pentameric form to monomeric form has been shown to occur in the surface of activated platelets (87). This dissociation process is supported by the activation of GPIIb/IIa (87). According to Bautista *et al*, CRP production by hepatocytes is also regulated by TNF-α causing atherosclerotic plaque and increased risk of high blood pressure resulting in hypertension (51). Moreover, CRP has been reported to display different immunoregulatory properties such as modulating platelets, opsonization, fixation of the complement system, enhancing leukocyte activity and mediation of LDL uptake by macrophages (19). CRP has also been discovered to be play active role in endothelial cells by inducing cellular adhesion molecule expression and inducing MCP-1 expression which induces

atherosclerosis (19). However, the characteristic of CRP is still yet to be discovered at pre-diabetes stage.

Additionally, fibrinogen is a complex plasma glycoprotein produced in the liver (88). This dimeric glycoprotein consists of three pairs of di-sulphide bonded polypeptide chains which are Aα, Bβ and the ɣ-chains (89). Fibrinogen plays a role in influencing plasma viscosity, deposition of fibrin, aggregation of platelets and has been recognised as a role player in atherosclerosis (88, 89). According to Dunn *et al,* thrombin activates fibrinogen during clot formation in atherosclerosis by cleaving two small peptides designated fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from the N-terminal parts of Aα-chains and Bβ-chains respectively (89). These activated fibrinogen molecules can then form a complex of fibrin and fibrinogen molecules (89).

Additionally, thrombin induces the formation of fibrin from fibrinogen (59). This step is then followed by cascade of events that contribute to clot formation (59). According to literature, the T2D display elevated levels of plasma fibrinogen have been linked to development of cardiovascular disease (21).This progression then cause more diabetics complications leading to cardiovascular diseases such as heart failure, stroke, angina. myocardial infarction (18).However the characteristic of fibrinogen remains unclear at pre-diabetes stage. P-selectin (s) and CD40L (s) has been shown to be the markers for platelet activation and aggregation in T2D (17, 56, 90, 91).

1.4.3. Soluble P-selectin and soluble CD40L as inflammatory markers

According to Carter et al, soluble P-selectin is the cell adhesion molecule responsible for attachment of leukocytes to the damaged endothelium and platelets (92). Soluble P-selectin is the marker for platelet activation (54). It is stored in the α granules of platelets and in endothelial cell Wiebel Palade bodies (92). Moreover, upon activation of platelets and endothelial cells

soluble P-selectin secreted (92). P-selectin plays different roles in the such as platelet rolling to the dysfunctional/damaged endothelium, supporting leukocyte rolling to the damaged endothelium and also platelet-leukocyte adhesion (92). Soluble P-selectin prevent adhesion to the vessel wall by binding to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. T2D and ischemic heart diseases has been shown to have elevated levels of P-selectin (s) (92). However, it remains unclear if pre-diabetics display the same characteristic of soluble P-selectin.

Soluble CD40L is the 35kDa glycoprotein from the TNF-α family (93, 94). According to Vaidyula *et al*, his pro-inflammatory member has been shown to be elevated in T2D (94). In circulation, it is stored and expressed in platelet surfaces (94). It confirms the firm binding of platelets to the damaged endothelial wall (54). Upon expression soluble CD40L interacts with cells that display its receptor CD40 for binding resulting in expression of adhesion molecules (94). It has been shown to interact with cells such as endothelial cells, monocytes and macrophages (94). However, it remains unclear if soluble CD40L is secreted by cells at pre-diabetic stage. In T2D activation of inflammatory signalling mechanisms contribute to the increase in inflammation and progression of T2D (48, 79)

1.5. Activation of c-Jun-N-terminal kinase (JNK) and inhibitor kappa B kinase (IKKβ*)*

Oxidative stress, hyperglycaemia, hyperlipidaemia and cytokines such as TNF-α and IL-6 cause activation of c-Jun-N-terminal kinase (JNK) and inhibitor kappa B kinase (IKKβ) (80, 95). This activation causes a serine phosphorylation of insulin receptor-1 (IRS-1) and insulin receptor-2 (IRS-2) which then impair the insulin signalling pathway (79, 80, 95). This result in insulin resistance (80). Interestingly this phosphorylation also causes translocation of NF-$_k$B and activator protein-1 (AP-1) from the cytoplasm to the nucleus inducing more inflammation which then contribute to progression of type 2 diabetes (48, 80). According to Zanergar *et al*,

CD40L ligation has also been shown to activate NF-$_K$B transcription factors which are known to be the major regulators of the immune and inflammatory response (93). However, it is still a debatable issue if this occur at a pre-diabetic stage. Henceforth, all these abnormalities have been discovered to be linked to consumption of an unhealthy diet as the primary source (96-98). High fat diets have been shown to have an effect on immunity due to insulin resistance (98).

1.6. High-fat diet and immunity

According to Sindhu *et al*, obese individuals and T2D have elevated levels of free fatty acids (FFAs) which has been shown to be related to immune function (99). Saturated fatty acids (SFAs) such as palmitate has been shown to show inflammatory effect on monocytes/macrophages and TLR signalling system (95, 99). FFAs has also been shown to activate the innate immune system via the TLR4 which is important in high-fat induced insulin resistance (99). Additionally, palmitate has been reported to induce pro-inflammatory cytokines such as TNF-α,IL-1β, IL-8 and IL-6 (99).

Kim *et al*, investigated the effect HFD using 2 experimental groups for a period of 8 weeks (98). The C57BL/6j mice were fed low fat diet (LFD) consisting of 10 kcal % fat and the second group fed HFD consisting of 60 kcal % fat (98). ELISA was used to test for the levels of TNF-α,IL-1β and IL-6 which was discovered to be elevated in HFD mice in comparison with LFD fed mice (98). Using immunoblotting ,they also discovered that there was an increase in TLR4,inducible NOS and activation of NF-$_K$B in the intestines of the HFD fed mice in comparison with the LFD fed mice (98). In conclusion to their research HFD was found to induce inflammation through TLR4 induction and NF-$_K$B activation resulting in increase in cytokines contributing to inflammation (95, 98). However, the effect of HFD is still a debatable

issue at the pre-diabetes stage. High-carbohydrate diet has also been shown to have effect on immunity due to insulin resistance.

1.7. High carbohydrate diet and immunity

High-carbohydrate diets induce elevated levels of glucose also known as hyperglycaemia (100). The consequences of hyperglycaemia has been shown to cause different mechanisms such increased flux through polyol pathway which contribute to oxidative stress (1). Activation of PKC due to hyperglycaemia can lead to increased diacylglycerol (DAG) since in a hyperglycaemic condition dihydroxy acetone phosphate become elevated and in some tissues, it is reduced to glycerol phosphate and converted to diacylglycerol (1). Activation of PKC-θ by DAG may cause insulin resistance in muscle through IRS-1 associated PI3K activity (1). Similarly, the activation of PKC-ε by DAG may cause insulin resistance to liver (100). However the high-fat high-carbohydrate diet has been discovered to be a factor contributing to pre-diabetes (101).

1.8. Justification of the study

The pre-diabetic model has been discovered using high-fat high-carbohydrate diet (50). This pre-diabetic model was discovered using values 6.1 to 6.9 mmol/L for impaired fasting glucose (IFG), 7,8 to 11.0 mmol/L impaired glucose tolerance (IGT) at two hour plasma glucose administration and glycated hemoglobin (HbA1c) of 5.7 % to 6.4 % considered pre-diabetic(50, 101). This glycated haemoglobin range is in line with the National Health and Nutrition Examination Survey (NHANES) data (50, 101).

Literature has shown that HFD cause insulin resistance in different mechanisms (25, 102-105). It has also been discovered that high-carbohydrate diet also causes insulin resistance in different mechanisms (1, 74). Both dietary groups (HFD and HCD) has been shown to also induce inflammation through activation of inflammatory signaling pathways such as NF-$_K$ B and

MAPKs (27, 79). Therefore, a combination of these two diets is hypothesised to induce more insulin resistance and result in more inflammation.

Literature has shown the effect of continuous consumption of unhealthy diet such as high-fat high-carbohydrate diet and living a sedentary lifestyle lead to T2D (96, 106, 107). Furthermore, according to literature it has also been shown that a long-lasting condition called pre-diabetes leads to T2D (50). The pre-diabetic model discovered in our laboratory mimicked the western diet consumed by human beings with sedentary lifestyle as these male pre-diabetic Sprague dawley rats were not exercising.

Additionally, literature has also shown that T2D have compromised immunity (1, 28). However, it remains unclear if a high-fat high-carbohydrate diet is the primary cause of the changes in quantity and functioning of immune cells. It also remains unclear if the immune activation occurs during progression of pre-diabetic stage to T2D. According to literature, it has been discovered that high-fat diet and high-carbohydrates diet cause secretion of inflammatory markers such IL-6 and TNFα (98, 108). Literature has also shown that inflammatory markers such as fibrinogen, CRP, CD40L, p-selectin, IL-6 and TNF-α are elevated in type 2 diabetic patients, but it still remains unclear if these inflammatory markers are secreted during progression of pre-diabetic stage. This study will characterise the clear understanding of the effect of this diet on neutrophils, monocytes, lymphocytes, eosinophils and basophils at pre-diabetic state in relation to changes that occur in T2D. Furthermore, there will be more clarity if there is secretion of the inflammatory markers (fibrinogen, CRP, CD40L, p-selectin, IL-6 and TNF-α) at pre-diabetic state.

1.9. Aim

The aim of this study is to characterise the changes in immune cell concentration during the progression of pre-diabetes to type 2 diabetes and accumulation of platelets in vessel wall at pre-diabetic stage in a diet-induced pre-diabetic rat model.

1.10. Objectives

To determine the response of immune cells after consumption of high-fat high-carbohydrate diet during progression of pre-diabetes to type 2 diabetes.

Evaluate the level of secretion of inflammatory markers at pre-diabetic stage.

To investigate if immune activation occurs during pre-diabetes stage

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CHAPTER 2

Prologue

Manuscript

Pre-diabetes has been shown to be caused by consumption of an unhealthy diet and living a sedentary lifestyle. Pre-diabetics have also been shown to be on a pre-diabetic stage for about 20 years before they become type 2 diabetic. However, combination of high-fat diet and high-carbohydrate diet has been shown to be the source of diet contributing to insulin resistance in pre-diabetics. Once they are type 2 diabetic, their immune system become compromised with elevated levels of IL-6, TNF-α, fibrinogen, C-reactive protein, P-selectin and CD40L which contributes to the progression of type 2 diabetes. Furthermore, type 2 diabetics develop complications due to dysregulated innate immunity such as atherosclerosis which then lead to cardiovascular diseases. However, it's a debatable issue if the immune activation and inflammation occur at the pre-diabetic stage. It's still a debatable issue if changes in immune cell concentrations and upregulation of inflammatory markers is a contributing factor of the progression of pre -diabetes stage to type 2 diabetes. Therefore, the present study evaluated if there are any changes in immune cell concentration in circulation during progression of pre-diabetes to type 2 diabetes in a diet-induced pre-diabetic rat model. It also evaluates if they are any immune cells in inflamed area and if there is any upregulation of the inflammatory markers such as IL-6, TNF-α, fibrinogen, C-reactive protein, P-selectin and CD40L at pre-diabetes stage in a diet-induced pre-diabetes rat model.

"The changes in immune cells concentration during the progression of pre-diabetes to type 2 diabetes in a diet-induced pre-diabetes rat model"

The current manuscript has been published in **Autoimmunity** (Impact factor 2.648) according to the journal's guidelines to authors (Appendix 1).

Title: The changes in Immune Cell Concentration during the progression of Pre-diabetes to Type 2 diabetes in a High-fat high-carbohydrate Diet-induced Pre-diabetic Rat Model.

Running title: Prediabetes Stage Immune Activation.

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ABSTRACT

Pre-diabetes is a long-lasting condition that precedes type 2 diabetes (T2D). T2D has been shown to suppress the immune response. However, it remains unclear if immune activation occurs before the onset of T2D during the progression of the pre-diabetic state. This study sought to characterise the changes in general immunity occurring during the progression from pre-diabetes to T2D. Male rats were fed a high-fat high-carbohydrate diet for 20 weeks (pre-diabetes induction period) and kept on the same diet being monitored for a further 12 weeks (experimental period). Blood was collected for haemocytometer analysis on week 0,4,8 and 12 of the experimental periods after which the animals were sacrificed. Plasma was collected from centrifuged blood for ELISA (TNF- α, CRP, P-selectin, CD40 L, fibrinogen & IL-6). Blood neutrophils percentage significantly decreased at week 12 possibly due to recruited neutrophils migrating to an inflamed area such as visceral adipose tissue as further observed. Due to hyperglycaemia there was significant increase in blood lymphocytes percentage at week 12. Blood monocytes percentage significantly increased at week 12. Monocytes recruited and circulated in blood due to hyperglycaemia for glucose uptake to decrease it from circulation. Blood eosinophils percentage significantly decreased at week 12. Eosinophils migrated to inflamed areas such as visceral adipose tissue as further observed. Blood basophils percentage significantly increased due to their recruitment and activation. TNF- α, CRP and IL-6 increased significantly after 12 weeks. There was also upregulation of fibrinogen, P-selectin and CD40L. The results of this study show that there are changes in immune cells concentration and that immune cells such as neutrophils and eosinophils migrate to inflamed areas such as adipose tissue. There is also upregulation of various inflammatory cytokines. Based on these findings, immune activation begins during the pre-diabetic state as there is upregulation of inflammatory markers**.**

Keywords: High-fat high-carbohydrate diet, immunity, Pre-diabetes, inflammation, type 2 diabetes

2.1. Introduction

According to the World Health Organisation, the global prevalence of type 2 diabetes (T2D) has risen from 4.7 % to 8.5 % among adults in the last 34 years (1, 2). In 2014 ,422 million adults were living with diabetes in comparison with the 108 million adults shown in 1980 (2). The pre-disposing factors are the continuous consumption of an unhealthy diet and living a sedentary lifestyle (1). Type 2 diabetic patients have been shown to have compromised immunity (3). Studies show that in type 2 diabetes there is an increase in the production of adipocytes and chemokines in the immune system due to cell dysfunction and insulin resistance (4). Obese individuals and rodents have been shown to be associated with chronic low-grade systemic inflammation called metabolic inflammation, which is another contributing factor that induces insulin resistance and T2D (2, 5-7). The chemokines and cytokines produced by adipose tissue have been shown to contribute to metabolic inflammation in obese individuals and rodents (6). However, according to Ahmad *et al*, free fatty acids that are circulating in blood of obese and T2D individuals have been shown to act on toll like receptors (TLR2 and TLR 4) to promote inflammatory signalling (7, 8). Additionally, in obese individuals, the imbalance between the free radicals and anti-oxidants caused by oxidative stress with the metabolic inflammation has been shown to also lead to risk of development of T2D (8). According to Graves and Kayal, hyperglycaemia causes disturbances of signalling factors (9). These include increased oxidative stress, elevated protein kinase C activation, shunting of the polyol pathway and increased formation of advanced glycation end products (9). Studies have shown that there is increased migration of neutrophils and simultaneous increases in lymphocyte activation (10, 11). Furthermore, monocytes are recruited and differentiate into macrophages which cause more secretion of cytokines for inflammation (12, 13). Additionally, eosinophils secrete cytokines that sustain macrophage activation causing additional secretion of cytokines for inflammation (14). This results in type 2 diabetic patients having increased levels of cytokines

like TNF-α and IL-6 (15, 16). Type 2 diabetic patients have also been shown to have elevated levels of C-reactive protein, fibrinogen, P-selectin and CD40 ligand (17-20). However, the profile of these markers is still remaining unclear at the pre-diabetic stage. The onset of T2D is usually preceded by a long lasting condition known as pre-diabetes which is characterized by glycated haemoglobin levels ranging from 5.7 % to 6.4 % (21). The effect of this condition on immune cells and various changes that occur during the progression to T2D are yet to be fully characterised. Recently, we have reported on a diet-induced pre-diabetic animal model that satisfies diagnosis as set out by the American Diabetes Association (21, 22). We have further shown that the continued exposure of the pre-diabetic animals to a high-fat high-carbohydrate diet for another 12 weeks result in the development of T2D (23). Based on this knowledge, this study sought to characterise the changes in the concentration of immune cells that occur during the progression from pre-diabetes to T2D.

2.2. Materials and methods

2.2.1. Drugs and Chemicals

All chemicals and reagents were sourced from the standard pharmaceutical suppliers and were of analytical grade. The materials and kits were sourced as follows: EDTA tubes (Greiner bio-one, Monroe, USA); Heparinized containers (Greiner bio-one, Monroe, USA); Glucose sticks (Mzansi Medical Supplies, Durban, South Africa); Rat TNF-α, CRP, P-selectin, CD40L, fibrinogen, glycated hemoglobin, IL-6 ELISA kits (Elabscience, Texas, USA).

2.2.2. Animal Studies

A group of male Sprague Dawley rats (150-180g) bred and kept at the Biomedical Research Unit (BRU) from the University of Kwa Zulu-Natal were used in the study. They were maintained under standard laboratory condition at temperature 22±2 °C and relative humidity

of (55±5%) and illumination (12h light/dark circle). The level of noise was maintained at less than 65 decibels and animals allowed access to food and fluids *ad libitum*. All animal procedures and housing conditions were approved by Animal Research Ethics committee of the University of Kwa Zulu-Natal (ethics no.: AREC/035/016M).

2.2.3. Induction of Pre-diabetes

Pre-diabetes was induced using a well-established laboratory protocol (21). Briefly, animals were divided into 2 groups at the beginning of an experiment. The first group animals were exposed to a high-fat high-carbohydrate diet supplemented with 15% fructose for 20 weeks (induction period). The second group of animals which was a control group was then fed standard rat diet for 20 weeks (21, 24). After 20 weeks, animals that exhibited the criteria according to American Diabetes Association with impaired fasting blood glucose concentration of 6,1 to 6.9 mmol/L, impaired glucose tolerance of 7.8-11.0mmol/L, and glycated haemoglobin of 5.7-6.4% were then considered to be pre-diabetic (21, 25).

2.2.4. ***Experimental design***

At the end of 20-week induction period, the animals were then divided into the non-diabetic control (NC) and the high-fat high-carbohydrate diet induced pre-diabetic group (PD) according to the results obtained from their health condition. Six rats (n=6) from the control group were used and another six rats were used from the pre-diabetic induced group (n=6). Both groups of rats were monitored for a further 12-week experimental period while carrying on with their diet. Parameters including neutrophils, lymphocytes, monocytes, eosinophils and basophils were measured every 4th week of the experimental period using a haemocytometer (Beckman Coulter, Indianapolis, United States). At the end of the experimental period the animals were sacrificed by being anaesthetised with Isofor (100mg/kg) (Safeline Pharmaceuticals (Pty) Ltd,

Roodeport, South Africa) *via* gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 minutes.

2.2.5. Blood and tissue collection

Blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers while the rats were unconscious. EDTA tubes were used to collect plasma for (TNF- α, CRP, P-selectin, CD40L, fibrinogen, glycated hemoglobin and IL-6) ELISA and lipid profile. The blood was centrifuged for 15 minutes at 1000 × g (2-8°C). Adipose tissue was collected for histology.

2.2.6. Oral glucose tolerance (OGTT) response

Both groups of animals were exposed to a 18-hour fasting period before OGT response and monitored during OGT test according to our established laboratory protocol (26). Briefly, after an 18-hour fasting period, glucose was measured (time 0) followed by loading of a monosaccharide syrup (glucose;0.86 g/kg. p. o) by oral gavage using an 18-gauge gavage needle that is 38mm long curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). For glucose concentration measurement, blood was collected using a tail-prick method and glucose concentrations were measured using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose concentrations were measured in different time intervals which are 15, 30, 60 and 120 minutes after carbohydrate loading orally.

2.2.7. Plasma glucose and triglyceride measurements

Plasma glucose and triglycerides concentration was measured using a tail prick method to collect blood. For blood glucose measurement, a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) was used, at the end of experimental period in both groups i.e. non-

diabetic (NC) and pre-diabetic group (PD). For triglycerides measurement, Accutrend Plus multi test kit (Roche diagnostics, Reinach, Switzerland) was used in every fourth week of experimental period.

2.2.8. Blood pressure measurements

Non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) was used to measure systolic, diastolic and mean arterial pressure (MAP). Both animal groups were monitored at week 0 of experimental period and week 12 which is the end of experimental period (21). The equipment was calibrated each day prior to measurements. The animals were kept warm at ± 30°C in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodlands Hills, California, USA) for 30 minutes before blood pressure recording. All measurements were conducted at 09h00.

2.2.9. Inflammatory marker measurements

TNF- α, CRP, fibrinogen, P-selectin, CD40L and IL-6 were measured using their respective ELISA kits from Elabscience as per manufacturer instructions. The optical density was determined of each well using a Spectrostar nanoplate spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany) at 450 nm.

2.2.10. Plasma lipid profile concentration measurements

At the end of experimental period, plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) concentrations were measured by the Global Clinical and Viral Laboratory (Amanzimtoti, South Africa). Friedewald's equation was used to calculate the LDL concentrations.

2.2.11. Histology of adipose tissue

Histological technique was performed according to the protocol which was modified based on the technique by Jali *et al.* Briefly, harvested tissue was fixed in formalin (fixative) and then dehydrated in alcohol (27). It was then cleared using xylene followed by embedding with molten wax to replace water and forming moulds with labelled cassettes for sectioning. Microtomy was used for sectioning followed by mounting of sections into slides that were kept overnight to dry (27). The slides were then cleared in xylene and then dehydrated in alcohol followed by haematoxylin and eosin (H&E) staining. Using an applicator, distyrene plasticizer xylene (DPX) mounting glue was dropped directly over the tissue section. An appropriate sized coverslip was then gentle lowered over the DPX ensuring no air bubbles are trapped. The slides were then left to dry overnight and then the photomicrographs were viewed on the digital light microscope using Leica application suit (version 3.4.0).

2.3. Statistics

Data is expressed as means ± standard error of means (SEM). Statistical analysis was conducted using Graph Pad Prism Instant Software (version 5.00, graph Pad Software, San Diego, California, USA). Two-way analysis of variance (ANOVA) was used to analyse differences between the controls and the experimental groups of immune cells at different time intervals while the unpaired t-test was used to analyse the results of inflammatory markers at the end of experimental period. Two-way ANOVA was followed by Bonferroni *post hoc* comparison test. Values of $p<0.05$ indicate statistical significance.

2.4. Results

2.4.1. Glycated haemoglobin (HbA1c) and mean arterial pressure

At the end of the induction and experimental periods, animals were sacrificed, and glycated haemoglobin was measured in both the non-diabetic control (NC) and pre-diabetic group (PD). The results showed that the HbA1c of PD was in the pre-diabetic range at week 20 and had increased to within the range of type 2 diabetes by week 32 (see Table 1). Table 1 also shows the mean arterial pressure at measured at week 20 and week 32 in both the non-diabetic control (NC) and pre-diabetic group (PD) to determine if there is any change in blood pressure during the pre-diabetic stage. The results show that the MAP significantly ($p<0.05$) increased at the end of induction period (week 20) as well as at week 32, which is the end of experimental period. [Insert table 1 here].

2.4.2. Plasma lipid profile

Table 2 shows the plasma lipid profile at the end of the experimental period (week 32) of both non-diabetic control (NC) and pre-diabetic group (PD) to determine the concentration of various types of plasma lipids in pre-diabetes. The results show that there was a significant ($p<0.05$) increase LDL with a concomitant significant ($p<0.05$) decrease in plasma HDL of the pre-diabetic group (PD) in comparison with a non-diabetic control (NC). [Insert table 2 here]

2.4.3. Plasma glucose and triglyceride levels

At the end of experimental period, plasma glucose and triglyceride levels were measured in both non-diabetic control (NC) and pre-diabetic group (PD). The results showed a significant ($p<0.05$) increase in plasma glucose levels of pre-diabetic group (PD) in comparison with a non-diabetic control (NC) as shown in figure 1a. Figure 1b shows a significant ($p<0.001$)

increase in triglycerides from week 4 to week 12 in the pre-diabetic group (PD) by comparison with the non-diabetic group (NC). (see figure 1b)

2.4.4. OGTT response

At the end of experimental period, both the groups NC and PD were exposed to 12 hours fasting and glucose was measured followed by the oral administration of monosaccharide syrup (glucose ;0,86 g/kg, p. o) at different time intervals (15, 30, 60 and 120 minutes) to assess glucose tolerance. The results showed a significant ($p<0.05$) increase in glucose concentration at 15, 30, 60 and 120 minutes in the pre-diabetic group (PD) by comparison with the non-diabetic control (NC) as shown in figure 2.

2.4.5. Neutrophil count

The experimental groups, NC and PD percentage count of blood neutrophils were monitored every fourth week of the experimental period (week 0, 4, 8, 12) to determine if there was any change in neutrophil concentration at the pre-diabetic stage. The results showed that the pre-diabetic group (PD) had a significantly ($p<0.05$) decreased blood neutrophil count at week 8 and showed more significant ($p<0.05$) decrease at week 12 of the experimental period by comparison with the non-diabetic control (NC) at the corresponding time intervals as shown in figure 3.

2.4.6. Histology of visceral adipose tissue

The visceral adipose tissue histology from the abdominal area of rats were performed for both non-diabetic control and pre-diabetic group at the end of the experimental period to observe for the presence of immune cells. The illustration from the photomicrographs obtained displayed that there were no immune cells found in non-diabetic control adipose tissue. However, the

photomicrographs of the pre-diabetic group showed neutrophil (image B) and eosinophil (image D) located in-between the adipocytes and infiltration sites spotted by pinkish stained cytoplasm (shown in image D) as shown in figure 4.

2.4.7. Lymphocyte count

The percentage count of blood lymphocytes was monitored in both the NC and PD experimental groups every fourth week of the experimental period (week 0, 4 ,8, 12) to determine if they are produced at pre-diabetic stage during immune response. The results showed that pre-diabetic group (PD) had a significantly ($p< 0.05$) increased blood lymphocytes count at week 12 of the experimental period by comparison with non-diabetic control (NC) at corresponding time intervals as shown in figure5.

2.4.8. Monocyte count

The monitored percentage count of blood monocytes in both the NC and PD experimental groups every fourth week of the experimental period (week 0,4,8,12) was measured to monitor if they are any produced monocytes produced and circulating in blood during immune response at pre-diabetes stage. Results showed that pre-diabetic group (PD) had a significantly ($p<0.05$) increased percentage count of blood monocytes at week 8 and 12 of the experimental period by comparison with the non-diabetic control. See figure 6.

2.4.9. Eosinophil count

The percentage count of blood eosinophils was monitored in both the NC and PD experimental groups every fourth week of the experimental period (week 0, 4, 8, 12). The results showed that pre-diabetic group (PD) had a significantly ($p<0.05$) decreased blood lymphocytes count at

week 12 of the experimental period by comparison with the non-diabetic control (NC) at the corresponding time intervals as shown in figure 7.

2.4.10. Basophil count

The percentage count of blood basophils was monitored in both the NC and PD experimental groups every fourth week of the experimental period (week 0, 4, 8, 12). The results showed that the pre-diabetic group (PD) had a significantly ($p<0.05$) increased blood lymphocyte count at week 12 of the experimental period by comparison with non-diabetic control (NC) at the corresponding time interval as shown in figure 8.

2.4.11. Inflammatory markers concentration

Plasma inflammatory marker concentration was measured in both experimental groups at the end of the experimental period. The results showed that PD had significantly ($p<0.05$) increased plasma levels of TNF-α, IL-6, CRP and P-selectin in comparison to NC. (see fig 9a, 9b, 9c, 9f). Figure 9d and 9e show an increase in fibrinogen and CD40L levels of PD by comparison with ND.

2.5. Discussion

Pre-diabetes is a long-lasting condition that precedes T2D (25). According to literature, type 2 diabetes compromises the immune system which is associated with the recruitment of immune cells and release of pro- inflammatory cytokines (28). This results in the recruitment of immune cells and release of pro-inflammatory cytokines (3, 29, 30). However, no studies have been done to characterise the changes in immune cell concentration during the progression of pre-diabetes to overt T2D. This study, therefore, was designed to focus on the changes that occur on immune cell concentration during pre-diabetes leading to the development of T2D.

In our laboratory, we have established a pre-diabetic rat model based on the American Diabetic Association criteria (21, 24, 25). After 20 weeks of pre-diabetes induction, the animals were observed for an additional 12 weeks to investigate the immunological changes that occur during the progression to T2D. Interestingly, after 12 weeks the haematological measure of glycated haemoglobin (HbA1c) increased to 6.6 % which indicated the progression to type 2 diabetes (25). Literature evidence suggests that hyperglycaemia leads to hypertension, hyperlipidaemia and inflammation contributing to type 2 diabetes (31). Furthermore, according to literature, it is hyperglycaemia that triggers the immune response even though it may not result in elevated levels of pro-inflammatory cytokines as compared to conditions in T2D (9).

However, according to the results of this study, there was an evident immune response and release of cytokines during the pre-diabetic state which is not characterized by hyperglycaemia but raised HbA1c. During this state, the cells have not completely lost their sensitivity to insulin, however, it is a slow progression that occurs due to the impairment of insulin signalling pathway (32, 33). Therefore, during pre-diabetes there is fluctuation of glucose levels as it progresses to fully impaired insulin signalling pathway resulting in insulin resistance (32, 34). According to Graves and Kayal, in diabetes, hyperglycaemia also contributes to metabolic and signalling abnormalities, however it has never been discovered if these abnormalities contribute to immunological changes during the pre-diabetes stage. Hyperglycaemia has been found to influence fluctuating concentrations of immune cells (35). In this study, we measured their concentrations at different time intervals through the progression of pre-diabetes to T2D. It has been discovered that neutrophils are the first line of defence and they migrate to an inflamed area in response to insulin resistance, lipotoxicity and glucotoxicity in T2D (10). Neutrophils then secrete pro-inflammatory cytokines such as TNF-α & IL-6 resulting in more inflammation and activation of NF-$_k$ B (10, 36). This then causes more insulin resistance through activation of serine residue of insulin receptor substrate thereby impairing the insulin signalling pathway

and causing more inflammation (37). Theoretically, expanding adipose tissue due to fat accumulation causes insulin resistance which attract neutrophils for inflammation (38). This correlates with the observed histology of adipose tissue of pre-diabetic group displaying neutrophil embedded in-between the adipocytes. During a hyperglycaemic state, the neutrophils form extracellular traps (36). After a high-fat meal, the neutrophils are recruited thus increasing their concentration in blood causing migration to adipose tissue (10). This correlates with the observed increase at week 4 showing that the neutrophils were recruited in bone marrow and circulating in blood and the subsequent decrease observed in blood from week 8 and week 12 showing that they were migrating to the inflamed areas such as adipose tissue as observed in histology obtained.

Lymphocytes such as B-cell mature to plasma cells and release more antibodies such as IgE antibodies in T2D patients which then bind to recruited basophils to activate them and secrete pro inflammatory cytokines (37, 39). This results in elevated levels of basophils and over-activated basophils in T2D. Denzel *et al* also mentioned that basophils are the main source of IL-4 and IL-6 in spleen and bone marrow after being stimulated by IL-3 and crosslinking with IgE receptors (39). The memory B-cell express IgG and IgE which allows them to recognise and react to free antigen (39, 40). The basophils then bind to IgE antibodies released and then over activated which correlates with the increase in concentration of basophils in pre-diabetic group at week 12 during progression of pre-diabetes. However, as the immune system becomes more compromised, more basophils will be recruited contributing to onset of T2D.

In this study, we observed that the concentration of lymphocytes increased in the pre-diabetic group at week 12 of the experimental period. We can speculate that the progression of insulin resistance triggers the immune system and recruitment of lymphocytes from the bone marrow. According to Patel *et al*, differentiation of B-cells into plasma cells require activity from antigen specific $CD4^+$ T cells. Additionally $CD8^+$ T cells promote adipose tissue macrophages

accumulation and pro-inflammatory cytokines gene expression (41). Due to high-fat high-carbohydrate diet, IL-6 is secreted which then stimulates differentiation of T and B cells, secretion of immunoglobulins, proliferation of thymocytes, and expression of acute-phase proteins in the liver (37). Furthermore, macrophages are classically activated and then secrete large amounts of pro-inflammatory cytokines and express high levels of costimulatory molecules which are required for T-cell activation (37, 42). Moreover these macrophages are potentially capable of presenting necrotic cell-derived antigens to T-cells and B-cells (42). This then activates adaptive immunity, resulting in clonal expansion of $CD4^{+}$Th1 cells and recruitment of $CD8^{+}$T-cells (37). This correlates with the increase in percentage count of blood lymphocytes of pre-diabetic group at week 12.

Theoretically monocytes secrete cytokines such as TNF-α and IL-6 which also activate the NF-$_{k}$B pathway causing chronic inflammation in T2D (37, 43). Moreover monocytes also differentiate into macrophages and then secrete more pro-inflammatory cytokines such as monocyte chemo attractant, TNF-α and IL-6 which cause chronic inflammation and progression of T2D (43). The results of this study showed that there was an increase in percentage cell count of blood monocytes in circulation during the pre-diabetic state. Insulin resistance in the adipose tissue result in secretion of cytokines such as IL-6, TNF- α and MCP-1 (12). During an immune response, IL-6 causes increase in the concentration of monocytes in blood (44). The changes in the immune system functioning that occur in in obesity and T2D has been shown to contribute to increased free fatty acid production (45). Saturated fatty acids such as palmitate found to be increased in obesity/ T2D has been shown to induce pro-inflammatory cytokines such as TNF-α,IL-6 and IL-8 (45). Additionally, saturated fatty acids activate toll like receptor 4 which then activate the JNK & IKKβ which activate inflammatory signalling pathway (37). According to Ahmad *et al*, in obese individuals ,long chain fatty acid including palmitate has been shown to synergistically contribute to increased levels of MCP-1 secretion and production when

combined with TNF-α (46). It has also been shown *in vitro* that MCP-1 can be secreted in various cells such as monocytes/macrophages and adipocytes when responding to the stimuli such as increased levels of TNF-α, free fatty acids and lipopolysaccharides (LPS) (46).This is also achieved by the TLR 4 -mediated signalling (45, 46). Moreover, obese individuals has also been shown that an interaction between LPS and TLR4 result in activation of different signalling mechanisms including AP1,JNK,MAPKs, ERK1/2 and NF-$_k$ B(46). MCP-1 then attract the monocytes to the macrophages (12, 47). The monocytes then migrate from the blood to the adipose tissue and macrophages where they differentiate into macrophages and secrete more cytokines (44, 47). Additionally, the recruited monocytes undergo the uptake of glucose to decrease glucose from circulation contributing to a decrease in immune response due to decreased in glucose concentration (44). However due to hyperglycaemia there is still recruitment of monocytes which correlates with the increase in percentage count of blood monocytes at week 12.

According to literature there is recruitment of eosinophils in T2D patients and secretion of cytokines such as IL-13, IL-6 and IL-4, where IL-4 sustains the alternatively activated macrophages (48). It is debatable whether the secreted IL-4 is secreted at pre-diabetic state and if it also sustains the alternatively activated macrophages at pre-diabetic stage to release more pro-inflammatory cytokines. According to Wu *et al,* eosinophils secrete cytokines such as IL-6, IL-4 and IL-13 (48) . Moreover the secreted IL-4 and IL-13 sustains alternatively macrophage activation of adipose tissue macrophages (48). They also maintain plasma cells in bone marrow (14). Therefore, based on the results we can speculate that the eosinophils migrate to the inflamed areas such as adipose tissue to sustain alternatively activated macrophages which then decreased their concentration in blood. This was further observed in the histology as it showed eosinophils embedded in-between the adipocytes.

According to literature, inflammatory cytokines such as CRP, TNF-α and IL-6 are elevated in T2D (30, 49). CRP has been shown to be the hepatokine that is associated with obesity and a potential risk factor for the development of T2D (50). CRP has also been shown to be linked with blood pressure and hyperlipidaemia contributing to progression of T2D (50). The results of the study show a significant increase in concentration at pre-diabetes implying contribution of T2D and cardiovascular complications. However ,TNF-α has been shown to be the key role linking obesity and T2D and has also been discovered to be a potent mediator/regulator of inflammation (50). According to Ahmad *et al*, it has also been discovered to play a role in inflammation and systemic insulin resistance (50). The results of this study show that there was a significant increase of TNF-α concentration at the pre-diabetic stage. TNF- α has been discovered to be increased due to insulin resistance caused by hyperglycaemia and hyperinsulinemia in monocytes and macrophages (51). However, the increase in activated macrophages have impact on release of nitric oxide by inducible nitric oxide synthase which results in the degradation of IRS-1 causing more insulin resistance thus increasing progression of pre-diabetes. By inducing adipocyte insulin resistance, TNF- α can also impair glucose uptake into adipocytes (52, 53). It also downregulates lipoprotein lipase expression in adipose tissue which can contribute to development of hyperlipidaemia which will lead to the development of T2D (53). IL-6 is secreted by most immune cells and was significantly increased by high-fat high-carbohydrate induced pre-diabetes. This cytokine (TNF- α) is one of the downstream target of NF-$_k$ B activation which promote a feed forward mechanism of inflammation (37, 54).

Additionally, T2D has been shown to have elevated levels of P-selectin, CD40 ligand and fibrinogen due to activation and accumulation of platelets in the damaged endothelium caused by oxidative stress and metabolic inflammation (55-57). In this study the increase in P-selectin indicated activated platelets and slight increase of CD40L indicate a beginning of the

accumulation of platelets in damaged endothelium. According to Linderman *et al*, in T2D P-selectin is secreted by activated platelets and CD40L confirms firm binding of platelets to the damaged endothelium (58). CD40L released further induce expression of pro-inflammatory cytokines in the damaged endothelium (58, 59). Furthermore, fibrinogen has been discovered to bind to receptors to platelet membrane during activated platelet aggregation (20, 55). In development of atherosclerosis, fibrinogen has been shown to form fibrin and fibrinogen degradation products which in turn bind LDL and stimulates cell proliferation (60, 61). However, we can speculate that in this study a slight increase in plasma fibrinogen concentration may indicate platelet aggregation and early characteristic of atherosclerosis development or cardiovascular risk factor.

2.6. Conclusion

In conclusion, chronic ingestion of a high-fat high-carbohydrate diet-induces pre-diabetes which then triggers an immune response. The immune response involves the recruitment of neutrophils, lymphocyte, monocytes, eosinophils and basophils. Immune cells such as neutrophils and eosinophils also migrate to inflamed areas such as visceral adipose tissue where they are imbedded in-between adipocytes. This then results in secretion of cytokines and increased secretion of TNF-α, CRP, CD40L, fibrinogen, P-selectin and IL-6 which possibly cause more immune response and inflammation due to activation of kinases that activate NF-$_k$ B and AP-1. The findings of this study warrant further investigations into the changes in the immune system during the development of type 2 diabetes from pre-diabetes in the high-fat high carbohydrate diet-induced animal model.

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2.8. Declaration of statement of interest

2.8.1. Competing interest-The authors declare no competing interests.

2.8.2. Funding-This work was funded by the National research foundation under Grant [number: 106041].

2.8.3. Ethics approval-All animal's procedures and housing conditions were approved by Animal Research Ethics committee of the University of Kwa Zulu-Natal (ethics no.: AREC/035/016M).

2.8.4. Availability of Data and Materials-The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

2.8.5. Author's contributions-NCM was involved in study design, conducted the experiments, analysed and interpreted data and was involved in writing the manuscript. PSN was involved in study design, analysed and interpreted data and was involved in writing the manuscript. AK was involved in conceptualization of the study, study design provided funding, the analysed and interpreted data and was involved in the writing of the manuscript. All authors have read and approved the final manuscript.

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2.10. Tables

Table 1: Glycated haemoglobin concentrations (%) and mean arterial pressure of both non-diabetic control (NC) in comparison with pre-diabetic group (PD) at the end of the experimental period. Values are presented as mean ± SEM.

	Week 20		Week 32	
Groups	**NC**	**PD**	**NC**	**PD**
HbA1c (%)	3.8±1.17	6.3±0.36*	4.3±0.26	6.6±0.98*
MAP (mmHg)	68.7±0.66	98.3±0.44*	72.0±0.96	107.3±0.89*

* = p <0.001 denotes comparison with ND.

Table 2: Plasma lipid profile of both non-diabetic control (NC) in comparison with pre-diabetic group (PD) at the end of the experimental period. Values are presented as mean ± SEM.

Groups	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
NC	4.04±0.032	1.22±0.031	1.70±0.026	2.40±0.045
PD	4.43±0.041	3.34±0.210*	0.85±0.012*	5.93±0.076*

* = p <0.05 denotes comparison with ND.

2.11. Figures and Legends

Figure 1

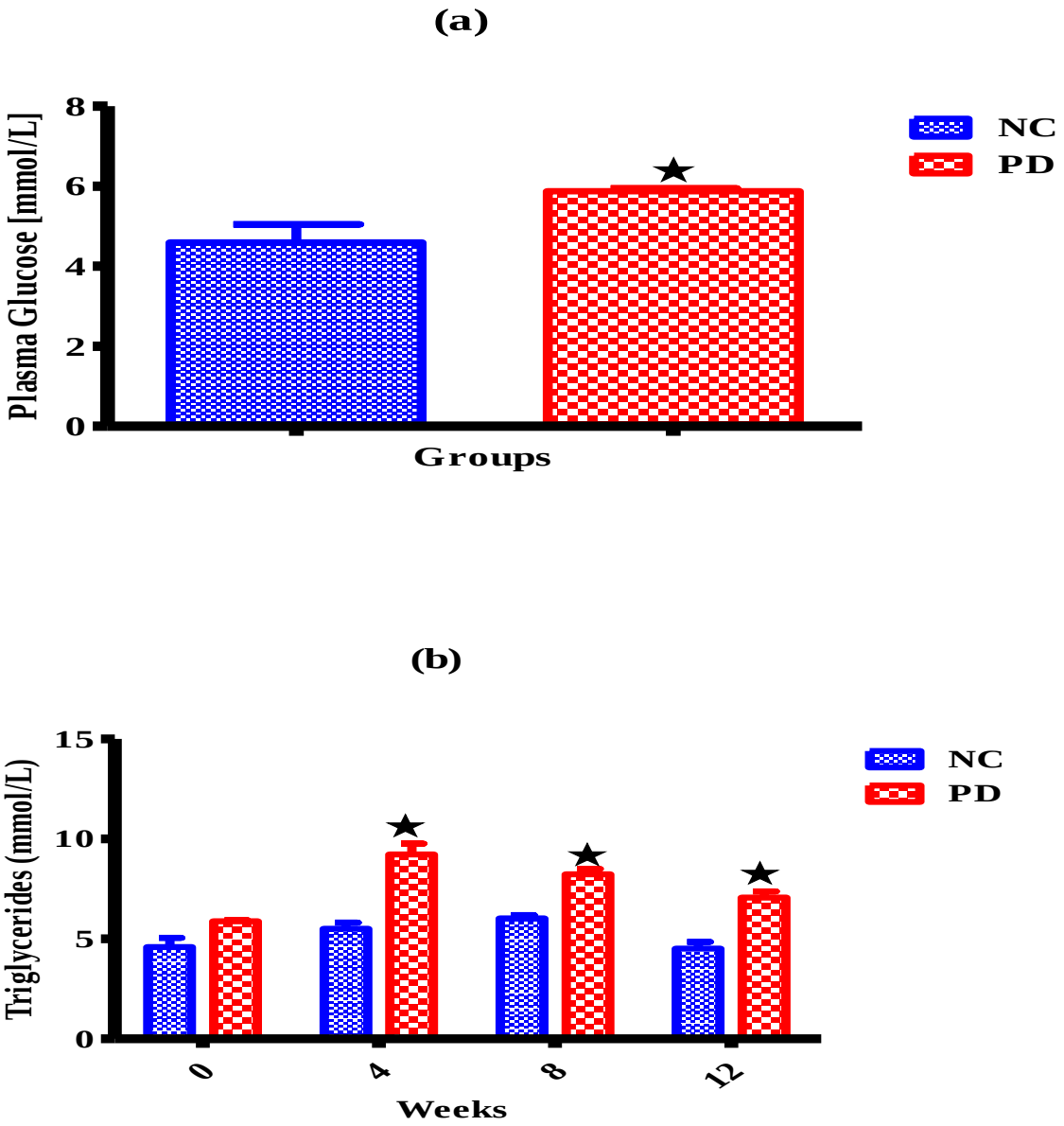


Figure 1: Plasma glucose levels at the end of experimental period (fig 1a) and the blood triglycerides during the progression of pre-diabetic stage (fig 1b) of non-diabetic control (NC) and pre-diabetic group (PD) at the end of experimental period. Values are represented as standard error of means (±SEM).★ =$p<0.05$ vs non-diabetic control.

Figure 2

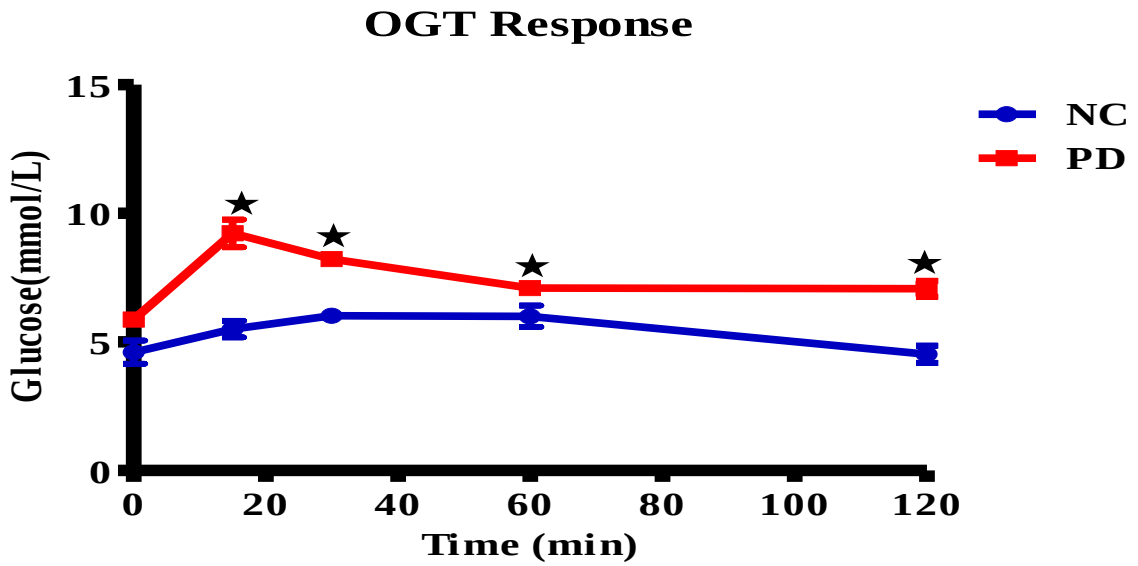


Figure 2: Oral glucose tolerance response of non-diabetic control (NC) and pre-diabetic group (PD) at the end of experimental period. Values are represented as standard error of means (±SEM).★ =p<0.05 vs non-diabetic control.

Figure 3

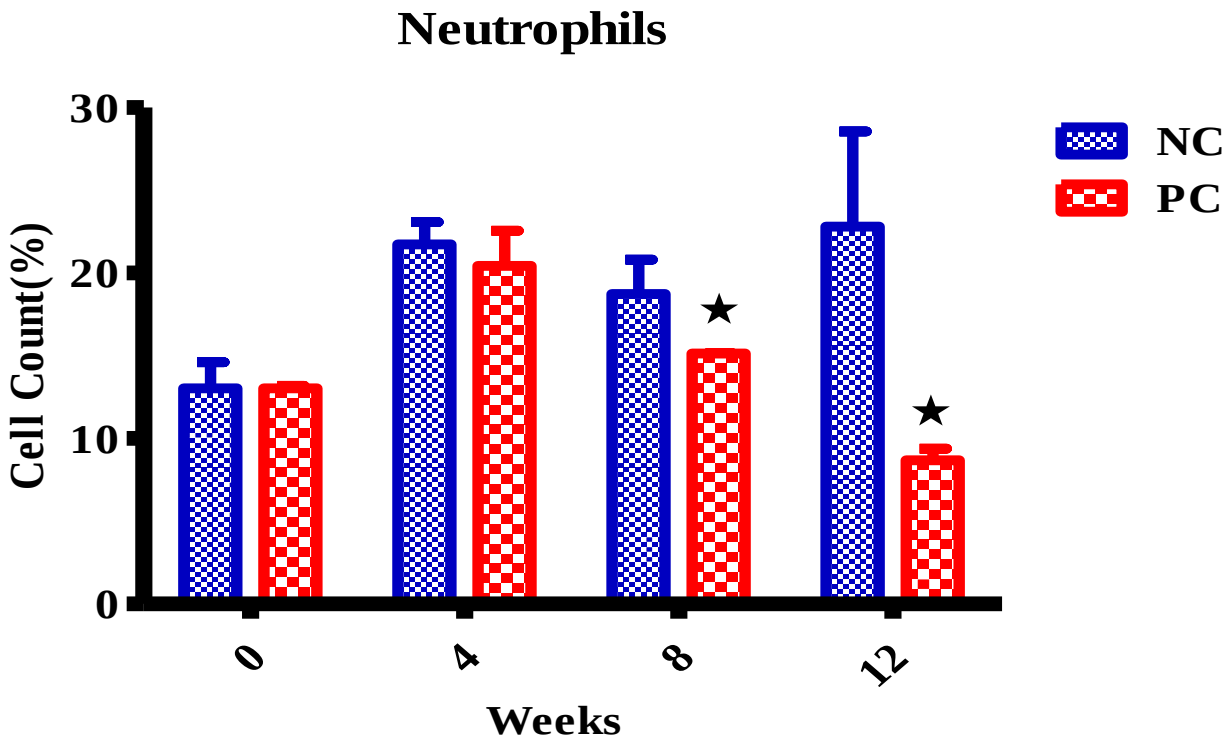


Figure 3: Blood neutrophil percentage count of non-diabetic control (NC) and pre-diabetic group (PD). Values are represented as standard error of means (±SEM). ★ =$p<0.05$ vs non-diabetic control.

Figure 4

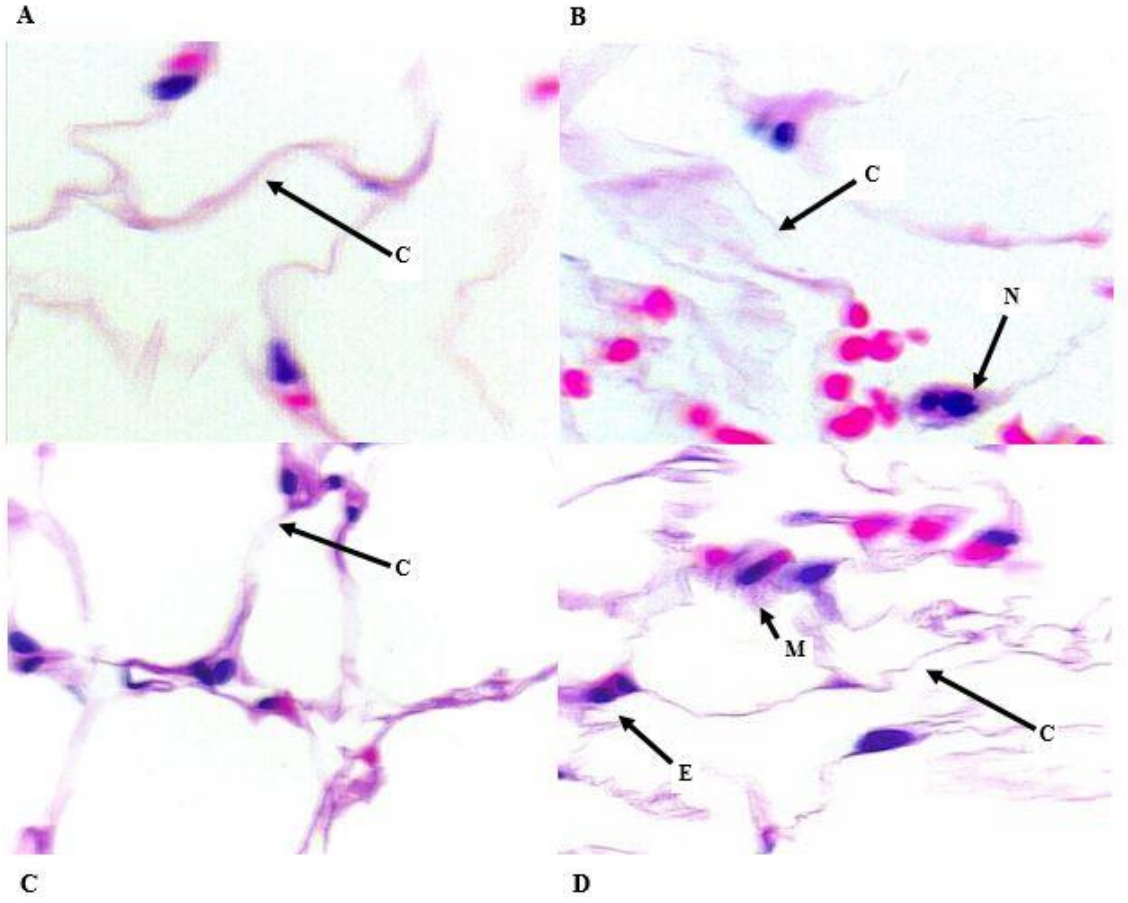


Figure 4: Photomicrographs illustrating the infiltration of neutrophil and eosinophils (immune cells) in adipose tissues due to inflammation in the non-diabetic control (A and C) compared with a pre-diabetic group (B and D) where C (cytoplasmic membrane) and N (neutrophil). Original magnification (×100) oil emulsion.

Figure 5

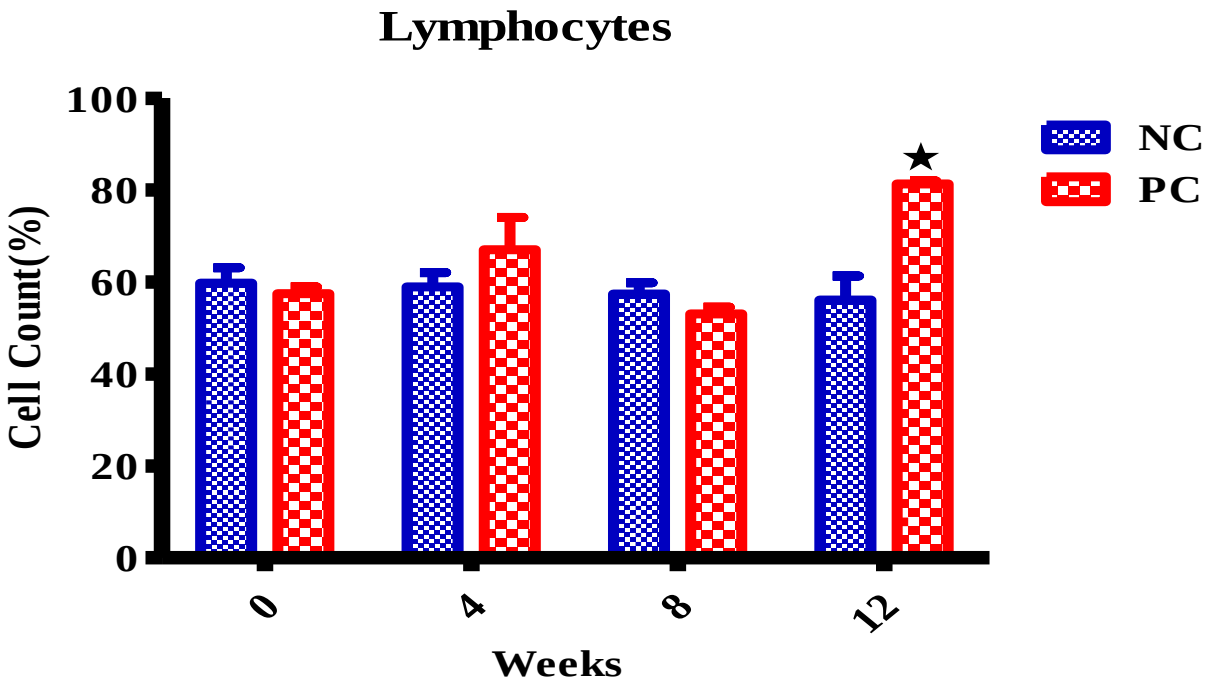


Figure 5: Blood lymphocytes percentage count of non-diabetic control (NC) and pre-diabetic group (PD). Values are represented as standard error of means (±SEM).★ =$p<0.05$ vs non-diabetic control.

Figure 6

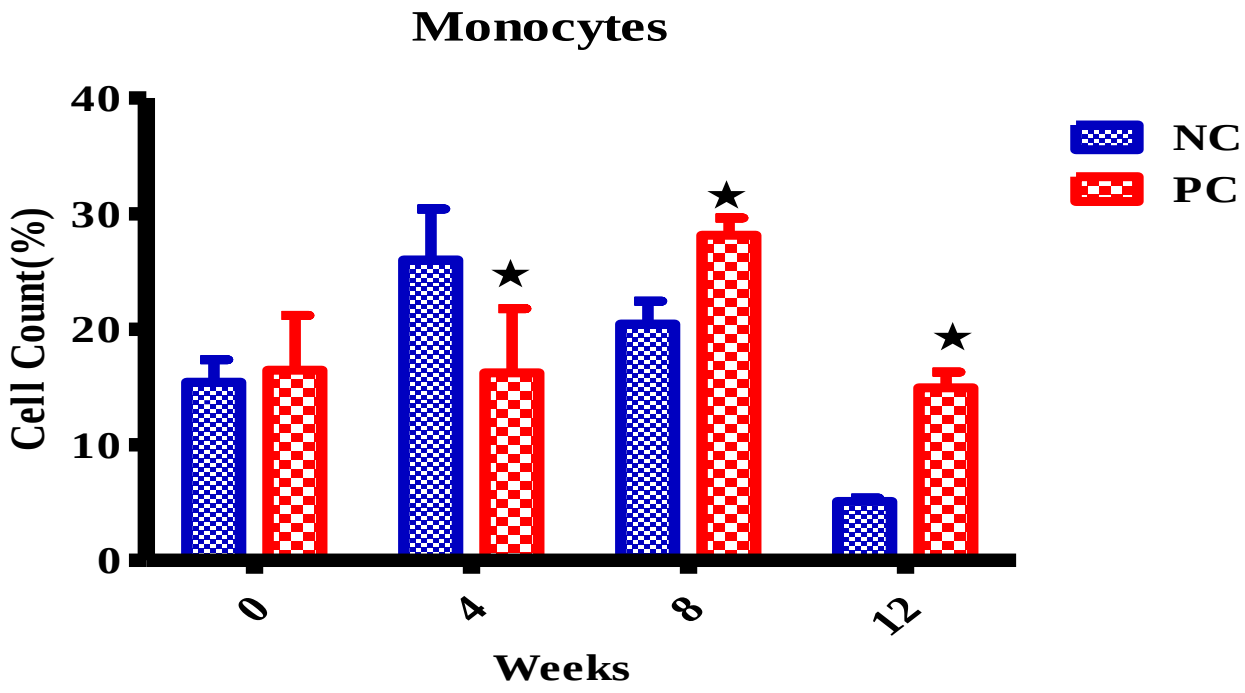


Figure6: Blood monocyte percentage count of non-diabetic control (NC) and pre-diabetic group (PD). Values are represented as standard error of means (±SEM).★=p<0.05 vs non-diabetic control.

Figure 7

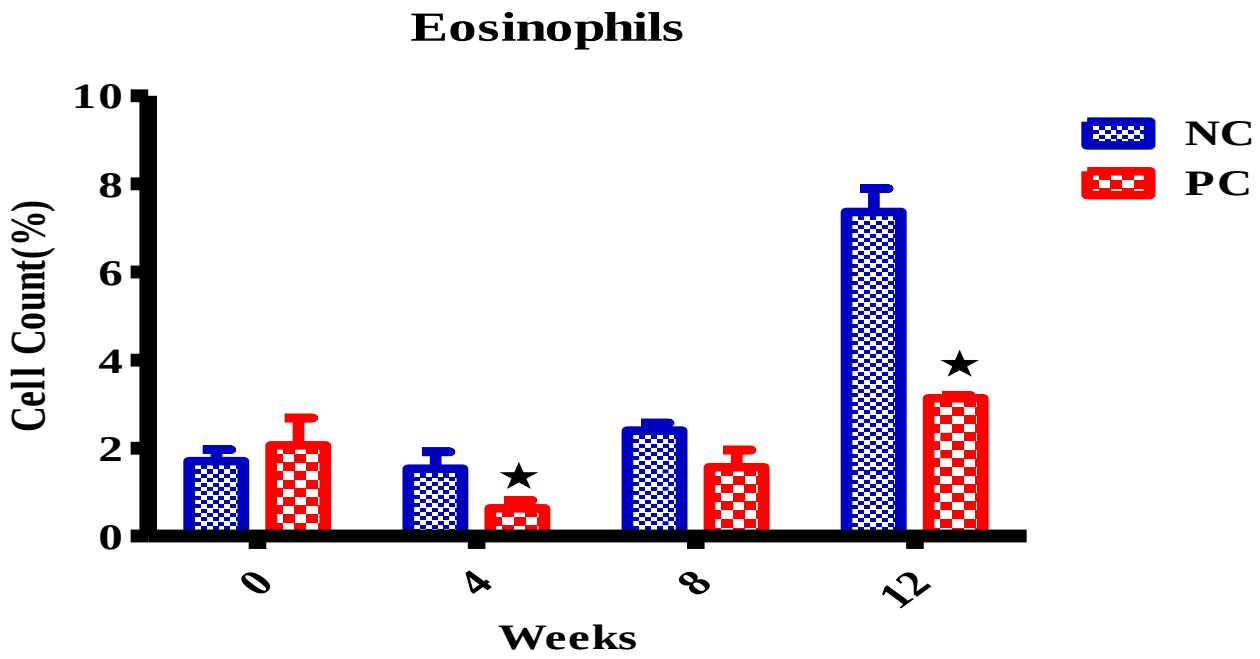


Figure 7: Blood eosinophils percentage count of non-diabetic control (NC) and pre-diabetic group (PD). Values are represented as standard error of means (±SEM).★=p<0.05 vs non-diabetic control.

Figure 8

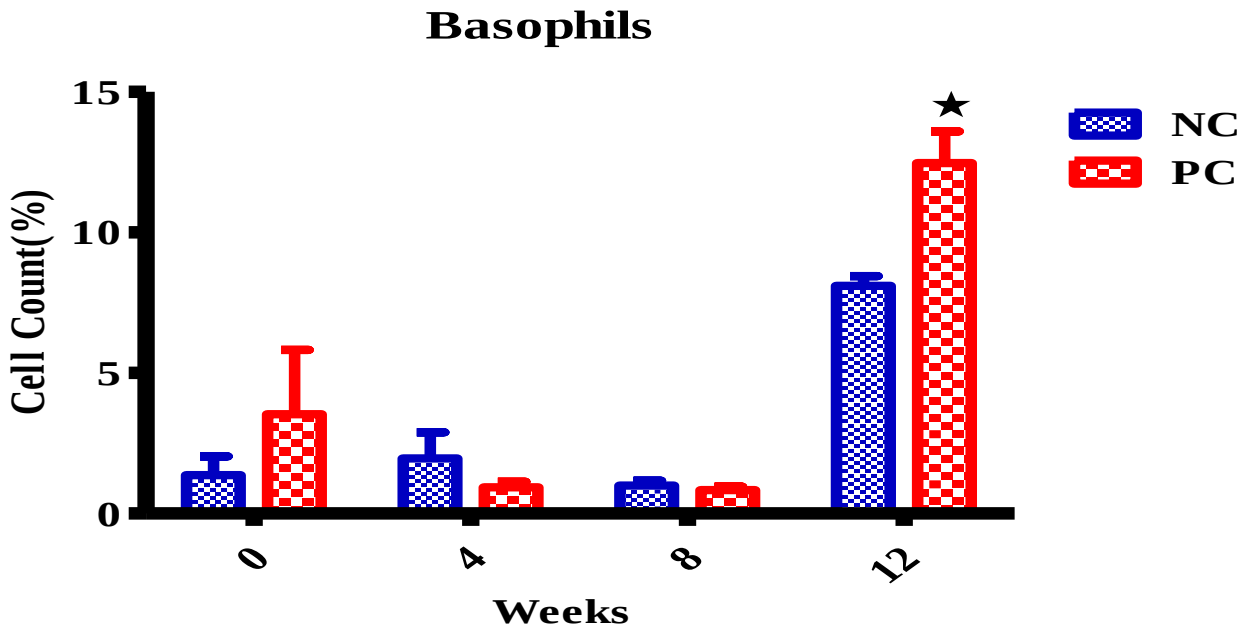


Figure 8: Blood basophils percentage count of non-diabetic control (NC) and pre-diabetic group (PD). Values are represented as standard error of means (±SEM). ★ =p<0.05 vs non-diabetic control.

Figure 9

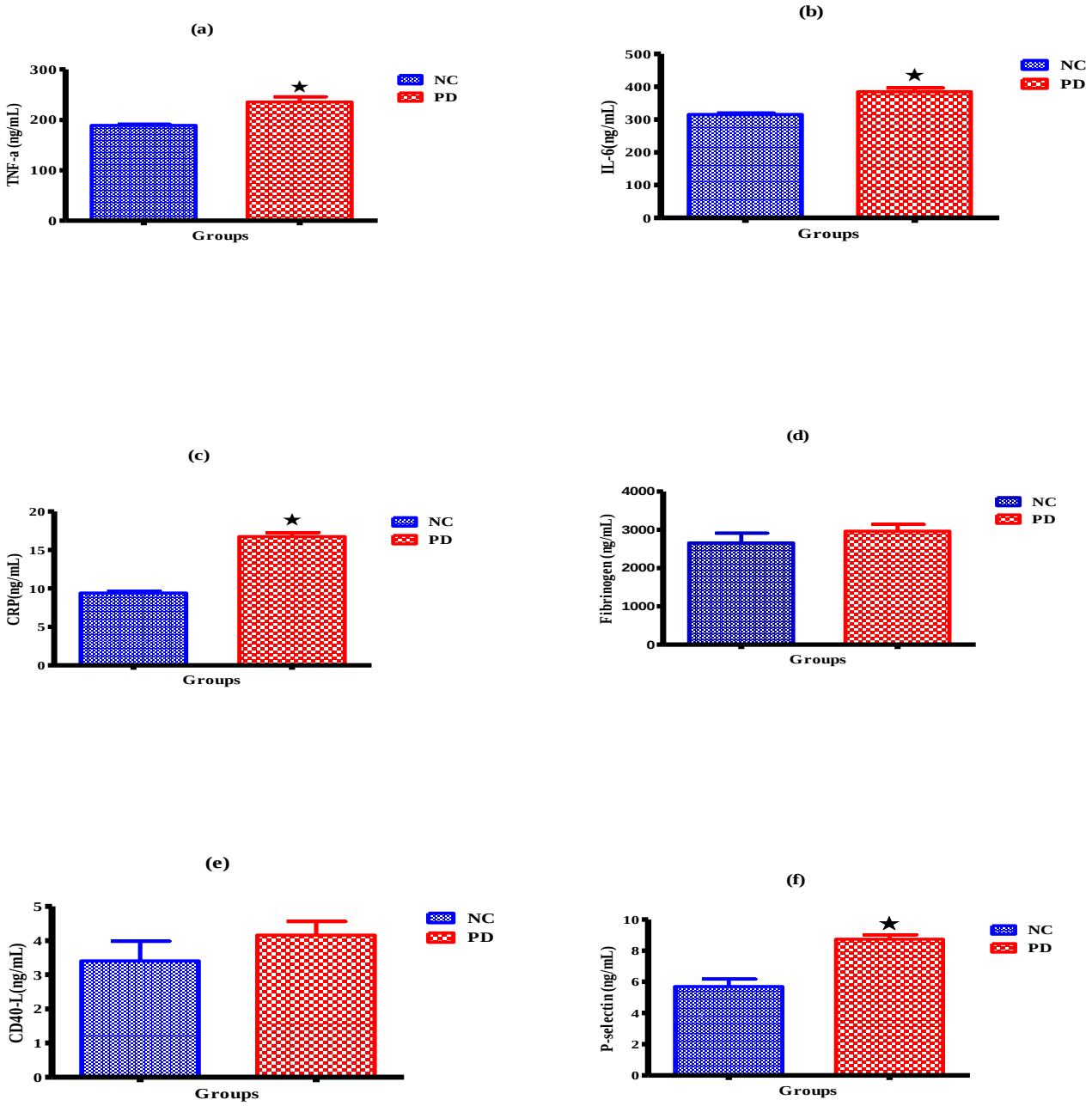
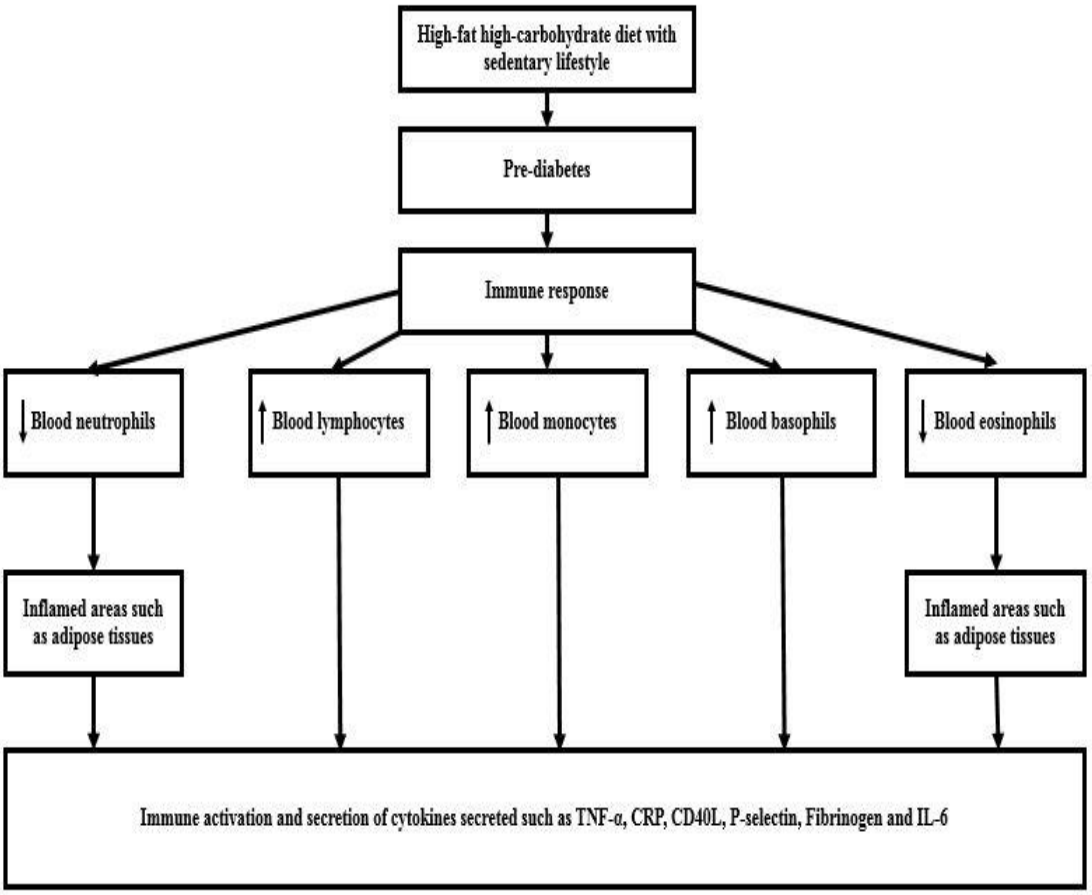


Figure 9: Inflammatory markers concentrations of non-diabetic control (NC) and pre-diabetic group (PD) after 12 weeks experimental period with TNF-α (a), IL-6 (b), CRP (c), fibrinogen (d), CD40-L (e) and P-selectin (f). Values are represented as standard error of means (±SEM). ★=p <0.05 vs non-diabetic control.

Figure 10


High-fat high-carbohydrate diet with sedentary lifestyle
Pre-diabetes
Immune response
Blood neutrophils
Blood lymphocytes
Blood monocytes
Blood basophils
Blood eosinophils
Inflamed areas such as adipose tissues
Inflamed areas such as adipose tissues
Immune activation and secretion of cytokines secreted such as TNF-α, CRP, CD40L, P-selectin, Fibrinogen and IL-6

CHAPTER 3: SYNTHESIS

Chronic ingestion of a high-fat high carbohydrate (HFHC) diet has been shown to induce pre-diabetes (1). Prediabetes has been shown to precede the onset of T2D. The animals in this study developed prediabetes after ingesting a HFHC diet for 20 weeks. This was shown by the increase in fasting plasma glucose levels and elevated levels of glycated haemoglobin. The animals also further displayed impaired glucose tolerance over the 2h OGT. Following a further 12 weeks of ingesting the HFHC diet, the plasma levels of glycated haemoglobin indicated that the animals had developed T2D. This was accompanied by a dysregulated lipid profile as shown by an increase in triglycerides, LDL and a decrease in HDL indicating hypercholesterolemia. T2D has been shown to have increased levels of triglycerides indicating dyslipidaemia due to accumulation of fats (2). T2D adipose tissue has been discovered to be one of the inflamed areas due to accumulation of fats which also cause insulin resistance (3). In this study, during the progression of pre-diabetes to T2D an increase of triglycerides levels every fourth week was observed. Moreover, in T2D accumulation of fats such as saturated fatty acids caused lipotoxicity which triggered an immune response (4). Additionally the elevated blood glucose levels over the experimental period may have triggered an immune response (5).

During immune response in T2D there is an increase in production of neutrophils which are the first immune cells to be recruited into an inflamed area such as in adipose tissue, damaged endothelium (6-9). An increase in circulating neutrophils concentration during progression of pre-diabetes stage indicated increased production from immune response. However, as the stage progressed there was a gradual decrease in neutrophils, and they were further observed in the histology of visceral adipose tissue dissected from the abdominal area of the rats.

T2D triggers an immune response resulting in an increase in lymphocytes concentrations (8, 10). B-cells mature to plasma cells and secrete antibodies such as IgE ,IgG (11). Additionally,

T-cells detect an antigen and secrete cytokines such as IL-6 (8). An increased in lymphocytes during progression of pre-diabetes stage (week 12) further indicated glucotoxicity. Monocytes play a huge role in T2D during immune response by facilitating the uptake of circulating glucose from the blood (10). Monocytes has been shown to have glucose transporters for glucose uptake to decrease it from circulation (10). Additionally, in T2D monocytes mature into macrophages and secrete pro-inflammatory cytokines such as TNF-α for inflammation (8, 10, 12). Furthermore, in T2D monocytes migrate to the intima due to dysfunctional endothelium and differentiate into macrophages which allow accumulation of LDL into foam cells contributing to atherosclerosis (13, 14). In this study there was an increase also an increase in monocytes at week 12. However, an increase at week 12 indicated that there was a certain number of monocytes that had matured to macrophages and the increasing amount was due to glucotoxicity causing more production of monocytes for the uptake of circulating glucose trying to decrease glucose to a normal level.

In T2D IgE antibodies bind to basophils that are produced during immune response since basophils display an IgE binding receptor to bind and activate the basophils (11, 15). This binding then activates basophils to secrete pro-inflammatory cytokines for inflammation which contributes to progression of T2D. An increase in basophil percentage count indicated the immune response and further secretion of cytokines for inflammation. The eosinophils has been shown to secrete cytokines for inflammation in the inflamed area in T2D (15, 16). Eosinophil are also produced due to hyperglycaemia in T2D (16). However they are then recruited to inflamed areas such as adipose tissue (16). In this study eosinophil blood percentage count occurred at week 8. However, there was a decrease in circulating eosinophil percentage count, indicating their recruitment to an inflamed area as it was further observed in a histology of visceral adipose tissue disserted from the abdominal area of the rat.

T2D abnormalities such as atherosclerosis has been shown to be due to inflammatory complications such as an increase in P-selectin and CD40L due to oxidative stress that damage the endothelium (17-19). Additionally T2D hepatocytes produce CRP which has also been shown to be elevated and also contributing to increased arterial pressure resulting in hypertension (20, 21). CRP, CD40L and P-selectin has been shown to be elevated at pre-diabetes stage. Additionally, an increased in mean arterial pressure was also observed in this study. T2D has also been shown do have elevated levels of inflammatory markers such as IL-6 and TNF-α (12, 21, 22). Hyperfibrinogenaemia is also another characteristic of T2D that contribute to cardiovascular abnormalities (23, 24). Fibrinogen, IL-6 and TNF-α contributing to inflammation and onset of T2D. In T2D it has been shown that oxidative stress, hyperglycaemia, hyperlipidaemia and cytokines activate NF-$_K$B and AP-1 contributing to more insulin resistance and inflammation.

CONCLUSION

Taken together, the results of this study show that chronic consumption of a high fat high carbohydrate diet leads to the development of prediabetes which, if continued, later develops into type 2 diabetes. The results also suggest that there is of immune activation during the prediabetes stage as evidenced by changes in concentration of immune cells at pre-diabetic stage and the upregulation of inflammatory markers contributing to inflammation and onset of T2D.

SHORTFALLS FUTURE STUDIES

The expression of the cytokines could not be measured in the inflamed areas such as in adipose tissue and the only targeted inflamed area was visceral adipose tissue. For future studies, expression of the cytokines in inflamed areas is recommended and histology of other inflamed areas to observe if the immune cells are recruited in other inflamed areas.

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APPENDIX

Autoimmunity guidelines to authors

Aims and scope

Current understanding of autoimmune disease is being increasingly underpinned by the new molecular sciences. Progress in this area has been little short of spectacular, and all clinical specialities now recognise autoimmunity as a major component of the diseases with which they are involved.

Autoimmunity is an international, peer reviewed journal that publishes articles of clinical and basic science on the pathogenesis, immunology, genetics, molecular biology, and treatment of autoimmune diseases. In addition to the basic mechanisms and elements of the immune system, the journal focuses on the autoimmune processes associated with systemic lupus erythematosus, rheumatoid arthritis, Sjogren syndrome, diabetes, multiple sclerosis, and other systemic and organ-specific autoimmune diseases. The journal is not restricted to any disease type or clinical speciality but reflects the areas where scientific progress is most rapid and clinical applications significant and widespread.

The journal is valuable to clinicians and researchers in immunology and molecular biology.

Instructions for authors

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UNIVERSITY OF KWAZULU-NATAL
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02 August 2016

Mr Mlindeli Gamede (213571877)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Gamede,

Protocol reference number: AREC/035/016M
Project title: Establishing a novel diet-based model for type 2 diabetes: Effects on selected renal parameters

Full Approval – Research Application

With regards to your revised application received on 15 July 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

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

Yours faithfully

.....

Prof S Islam, PhD
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/ms

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